**TESIS DOCTORAL**

**María Isabel Pozo Romero**

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# YEASTS in FLORAL NECTAR:
## community ecology and interactions with insect pollinators and host plants

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# Yeasts in floral nectar: community ecology and interactions with insect pollinators and host plants

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**Yeasts in floral nectar:**

**community ecology and interactions with insect pollinators and host plants**

Levaduras en el néctar floral: ecología de comunidades e interacciones con insectos polinizadores y plantas hospedadoras

Memoria presentada por **María Isabel Pozo Romero** para optar al grado de Doctor por la Universidad de Sevilla

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*A mi familia.*
*A Alfredo.*

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# Índice de contenidos

# Introducción general

*"Las relaciones entre plantas y animales no parecen ser tan simples como vienen a ilustrar los ejemplos clásicos de plantas y polinizadores. Quizás sea más frecuente la participación de un tercer convidado."*

(Carlos M. Herrera)

### *Interacciones, ecología y evolución*

Desde el pasado siglo, la consideración de la *interacción planta – animal,* y no solo el estudio de estos dos reinos por separado, botánica y zoología, ha permitido importantes avances en los campos de la ecología y la evolución. Bajo este enfoque, se ha logrado entender mejor las relaciones ecológicas entre plantas y animales, y cómo estas relaciones, a su vez, han supuesto un potente motor de diversificación para ambos grupos (Herrera y Pellmyr, 2002). Por ejemplo, es conocida la existencia de complejas interacciones, de carácter mutualista y antagonista, entre plantas y herbívoros, plantas y frugívoros, o plantas y polinizadores. En concreto, la interacción planta-polinizador se presenta como un sistema binario, de carácter mutualista, en el que las sésiles plantas obtienen los beneficios de la reproducción cruzada, y los polinizadores pueden obtener valiosos recursos alimenticios, polen y néctar principalmente.

Pese a la importancia de los mutualismos como factores para la estructura de las comunidades, y como factores generadores de biodiversidad (Kaiser-Bunbury *et al*., 2010), en los últimos años se cuestiona si el enfoque binario de interacción y adaptación bidireccional, como el aplicado a plantas y polinizadores, es suficiente para explicar de manera satisfactoria el funcionamiento y la evolución de los organismos en los sistemas naturales. En este sentido, existe una evidencia creciente en torno al papel activo que "ladrones" y parásitos desempeñan en muchos mutualismos (Bronstein *et al*. 2006). Por ejemplo, uno de los ejemplos más clásicos de sistema tripartito entre plantas e insectos lo protagonizan ciertos grupos de hemípteros parasitoides en la asociación de índole mutualista que tiene lugar entre acacias y hormigas en función defensiva en regiones tropicales (Ueda *et al*., 2008). Otras relaciones ternarias entre las plantas y sus polinizadores se establecen por la inclusión de hormigas nectarívoras, las cuales introducen, y consumen finalmente,

levaduras en el néctar (Junker *et al.*, 2007; de Vega y Herrera, en prensa). El estudio de interacciones entre plantas y distintos grupos de organismos, debe asimismo tener en cuenta que muchos de los rasgos funcionales de las plantas están influenciados por la presencia de microorganismos, ya sea por parte de simbiontes endofíticos o epifíticos (revisado en Friesen *et al.*, 2011). Así, otros ejemplos de interacciones tripartitas, descritos recientemente, incluyen a microbios como participantes, como el triángulo planta-micorriza-bacteria (Sarabia *et al.*, 2010), o planta-micorriza-virus (Herrero *et al.*, 2009). Esta tesis analiza las interacciones entre plantas, polinizadores y levaduras del néctar floral, una interacción tripartita de gran interés para el éxito reproductivo de las plantas que, sin embargo, ha pasado desapercibida durante muchos años, incluso en aquellos años de florecimiento de la biología de la polinización, en la segunda mitad del siglo XX.

***Néctar e interacción planta-polinizador***

En la interacción mutualista entre las plantas y sus polinizadores, el néctar es la forma de recompensa más frecuente (Simpson y Neff, 1983). Por ello, los estudiosos de la polinización han realizado grandes esfuerzos para estudiar sus características -principalmente su fisiología y patrones de secreción, concentración, volumen y composición de azúcares mayoritarios-. Algunos rasgos del néctar han sido considerados tradicionalmente como variables (como el volumen o la concentración), mientras que otros, como la composición de azúcares, se han considerado rasgos uniformes y evolutivamente conservados para las especies. Concretamente, se pensaba que la fuerte selección ejercida por los polinizadores, en función de sus requerimientos energéticos y demás características morfológicas y de conducta, era la que fijaba unas determinadas condiciones en la recompensa floral (p.ej. Wykes, 1952; Percival, 1961; Baker y Baker, 1983, 1986; Feinsinger, 1983; Elisens y Freeman, 1988; de la Barrera y Nobel, 2004; Galetto y Bernardello, 2004; Nicolson y Thornburg, 2007). De este modo se lograba explicar, en bastantes casos, el hecho de que especies con un origen filogenético dispar llegasen a presentar convergencia en los rasgos del néctar, por lo que este enfoque fue elevado a paradigma en la década de los 80. No obstante, en los últimos 30 años se han

señalado diversas limitaciones de este enfoque (por ejemplo, Herrera, 1996). Entre éstas, que la mayor parte de las especies de plantas son visitadas por diversos grupos de polinizadores (Herrera, 1987a; Gómez, 2002), o que sólo una fracción de los visitantes florales efectivamente realizan el servicio de polinización. Sin embargo, la constancia en la composición del néctar al nivel de especie, que constituye uno de los elementos de la teoría de los "síndromes florales" (Fenster *et al.*, 2004), se ha cuestionado más recientemente, al demostrarse una gran variabilidad intraespecífica en la composición del néctar para una especie de planta en el Sur de España (Herrera *et al.*, 2006).

Factores ambientales y genéticos pueden provocar diferencias a nivel intraespecífico para diversos componentes del néctar, como patrones de secreción, concentración, volumen, y composición (Nicolson *et al.*, 2007). Sin embargo, en los últimos años se ha demostrado para un número creciente de especies que, en relación a concentración y composición de azúcares mayoritarios, la variabilidad intraespecífica se presenta principalmente dentro de plantas individuales, y está directamente relacionada con la contaminación microbiana del néctar que tiene lugar en condiciones naturales (Herrera *et al.*, 2008). La relación de causalidad entre la presencia de levaduras en el néctar y las modificaciones acontecidas en cuanto a la composición química del mismo, se demuestra experimentalmente al comparar los azúcares presentes en el néctar de plantas visitadas por insectos, respecto a otras plantas de la misma especie en las que se han impedido estas visitas (Canto *et al.* 2007, 2011). Asimismo, se observa que la transferencia de levaduras hacia el néctar sucede a partir del contacto con las probóscides de ciertos insectos (Brysch-Herzberg, 2004; Canto *et al.*, 2008). Las levaduras pueden llegar a ser extremadamente abundantes en el néctar, y recientemente se ha descrito su presencia en numerosas especies de plantas, en diversas y remotas localizaciones geográficas de varios continentes (Herrera *et al.*, 2009, de Vega *et al.*, 2009; Belisle *et al.*, 2011). Así, al introducir a las levaduras en la interacción planta-animal, unidas a través de la principal recompensa floral, el néctar, encontramos un triángulo biológico cuyo resultado neto es difícil de predecir con los datos disponibles.

***Las levaduras del néctar floral***

Desde hace más de un siglo, estudios microbiológicos clásicos han puesto de manifiesto la presencia de levaduras en las flores (Boutroux, 1884, Schuster y Úlehla, 1913; Grüss, 1917; Schoellhorn, 1919; Guilliermond, 1920; Jimbo, 1926; Nadson y Krassilnikov, 1927). Principalmente por limitaciones técnicas, estos primeros estudios no distinguen las levaduras del néctar floral, o incluso aquellas asociadas al polen, de las habitantes de la superficie de la corola. En las últimas décadas del siglo XX empieza a citarse la presencia ocasional de microorganismos -y más concretamente, de levaduras- en el néctar floral, en este caso por estudiosos de los sistemas de polinización (Baker y Baker, 1975, 1983; Gilliam *et al*., 1983; Kevan *et al*., 1988; Eisikowitch *et al*., 1990 a, b; Kearns y Inouye, 1993; Ehlers y Olesen, 1997). Incluso, alguno de estos trabajos advierte sobre el posible papel de estos microorganismos en la degradación del néctar floral, como por ejemplo en Baker y Baker (1983). A pesar de estas menciones ocasionales, las publicaciones sobre fenómenos de polinización que se dedican directamente a las levaduras nectarívoras han sido tradicionalmente muy escasas (Gilliam *et al*., 1983; Sadhu y Waraich, 1985; Kevan *et al*., 1988; Eisikowitch *et al*., 1990a, b; Lawton *et al*., 1993).

Particularmente llamativa es la carencia de datos cuantitativos sobre la frecuencia de la contaminación por levaduras en los néctares florales y la densidad de levaduras alcanzada por unidad de volumen de néctar, a pesar de la simplicidad de estas medidas, y de que, al consumir las levaduras los azúcares presentes en el néctar, estos datos se relacionan directamente con la calidad del néctar como recurso (Herrera *et al*., 2008). Incluso en la literatura microbiológica más reciente, en la que pueden hallarse con cierta frecuencia trabajos sobre levaduras florales, son muy escasos los datos sobre frecuencia y abundancia de las levaduras del néctar en condiciones naturales (ver Sandhu y Waraich, 1985; Brysch-Herzberg, 2004). Son aún escasos los estudios sobre posibles factores explicativos de los valores de frecuencia y abundancia de levaduras hallados en el néctar, exceptuando el caso concreto de la temperatura, cuya influencia sobre ambas variables ha sido demostrada (Brysch-Herzberg, 2004). Tampoco aparecen medidas de incidencia de

levaduras en el néctar en las últimas revisiones aparecidas sobre néctar y ecología floral (Spencer y Spencer, 1997; Rosa y Péter, 2006; Nicolson *et al.*, 2007).

En cuanto a la diversidad de especies de levaduras que habitan el néctar floral, se han aislado diversas especies en función de la región y la especie de planta hospedadora, si bien éstas se asocian principalmente a los ascomicetos *Candida* y *Metschnikowia* (Sandhu y Waraich, 1985, Herzberg *et al.*, 2002, Brysch-Herzberg, 2004, Mushtaq *et al.*, 2006; Mushtaq *et al.*, 2007). Por su alta concentración de azúcares, y la presencia de proteínas y aminoácidos (Baker y Baker, 1983), el néctar podría considerarse un medio rico para el desarrollo microbiano, pero en realidad impone ciertas restricciones al crecimiento de los microorganismos. Entre las restricciones más relevantes para su colonización se encuentran su escasa disponibilidad temporal y la necesidad de vectores para su colonización. Otros factores, como son la alta osmolaridad y la presencia, en muchas especies, de compuestos del metabolismo secundario con actividad fungicida podrían limitar, subsiguientemente, la fase de establecimiento de estos microbios (Adler, 2000; Herrera *et al.*, 2010). A pesar de que los hongos son organismos especialmente ubicuos, las condiciones específicas del néctar podrían requerir de una adaptación diferencial por parte de las levaduras nectarívoras ante estas restricciones, si bien esta hipótesis permanece sin comprobar hasta la fecha de finalización de esta tesis.

Diversos insectos, entre los que se incluyen especies de escarabajos, abejas, y moscas, albergan levaduras en sus piezas bucales y aparato digestivo (Gilbert, 1980; Guilliam, 1983; Lachance *et al.*, 2001; Rosa *et al.*, 2003; Suh *et al.*, 2005; Canto *et al.*, 2008). Como se ha señalado anteriormente, son estos insectos los que transfieren las levaduras desde sus piezas bucales hacia el néctar. Sin embargo, se desconoce el efecto de la presencia de levaduras en el néctar para el comportamiento de los insectos como consumidores finales del recurso, a excepción del trabajo de Kevan *et al.* (1988) con *Apis mellifera,* ensayo en el cual no se detectó comportamiento diferencial respecto a la presencia de levaduras en el néctar.

## Organización de la tesis

En los tres primeros capítulos de esta tesis se aborda el estudio cuantitativo y cualitativo de las levaduras nectarívoras mediante el análisis microscópico y microbiológico del néctar floral. El perfil químico del néctar es un rasgo con base genética (Mitchell, 2004), por lo que sólo por razones filogenéticas podría originarse una fuente de variación interespecífica en la incidencia de levaduras del néctar. Por lo tanto, en primer lugar se valorará la generalidad de la presencia microbiana en el néctar floral para 105 especies de planta en el Parque Natural Sierras de Cazorla, Segura y las Villas durante tres años consecutivos (2008-2011) (Capítulo 1). Estos datos permiten entrever patrones de variación intraespecífica para la incidencia de levaduras del néctar en las diversas especies, lo cual plantea la necesidad de un estudio más detallado de patrones de distribución de las levaduras para el néctar de una única especie (Capítulo 2). En este capítulo, se estudia la incidencia de los factores espaciales y temporales, así como la variación existente entre plantas y dentro de plantas (flores dentro de plantas, entre nectarios de una misma flor) en la incidencia de levaduras en el néctar de *Helleborus foetidus*. Determinar el porcentaje de la variación total que tiene lugar dentro de individuos es crucial para entender las posibilidades de los polinizadores para ejercer selección sobre las características del néctar capaces de influir sobre el crecimiento de las levaduras, pudiendo ser éste un ejemplo más de "multiplicidad en la unidad" en el cual la selección fenotípica fuera dificultada por la alta variación existente dentro de cada individuo (Herrera, 2009). En el siguiente capítulo (Capítulo 3), se identifican por métodos moleculares las especies de levadura integrantes de las comunidades nectarívoras en nuestra zona de estudio, valorando la riqueza en especies de las comunidades de levadura que se constituyen, ya sea al nivel de las flores individuales como al nivel de las especies de plantas.

Un segundo bloque dentro de la organización de esta tesis doctoral lo componen los Capítulos 4 y 5, de carácter más experimental, orientados a analizar mecanismos y procesos en la interacción ternaria planta-polinizador-levadura. En el Capítulo 4 se estudian los mecanismos que posibilitan la contaminación del néctar

con levaduras, mediante la comparación de la comunidad de levaduras asociada a las probóscides de insectos polinizadores (procesos de inmigración) y la comunidad de levaduras finalmente presente en el néctar de las especies a las cuales visitan estos insectos, es decir, el conjunto de levaduras supervivientes a diferentes restricciones del néctar, tales como disponibilidad del recurso, alta osmolaridad y presencia de compuestos secundarios potencialmente tóxicos. También se valorará la importancia de otras posibles fuentes de levaduras para el néctar, como aire y filoplano. Finalmente (Capítulo 5), se espera que la contaminación microbiana del néctar pueda influir en los polinizadores, ya que éstos visitarán a partir de ese momento flores con microorganismos en su néctar, convertido por la vía del metabolismo microbiano en un recurso más variable y de menor recompensa. En este capítulo se investigará si esta nueva situación es detectada por los insectos y puede por lo tanto afectar a su elección floral.

### Área de estudio y especies modelo

Todo el trabajo de campo asociado al desarrollo de esta tesis doctoral se ha realizado en las Sierras de Cazorla, Segura y las Villas, en el extremo oriental de la provincia de Jaén, en el sureste de la Península Ibérica. Los macizos montañosos que conforman estas sierras son de naturaleza básica, y pertenecen al sistema de cordilleras prebéticas (Estrella, 1979), situado en el extremo oriental de la depresión del Guadalquivir (Fig. 1a). Sus picos más altos rondan los 2000 m.s.m., y sus zonas bajas apenas superan los 500 m.s.n.m. (Soriano, 1987). La zona de estudio presenta clima mediterráneo con cierto matiz continental, caracterizado por veranos cálidos y secos, y temperaturas muy suaves y precipitaciones por encima de la media de la región durante el resto del año. Sin embargo, a causa de la influencia de su marcado relieve, en la zona se definen diversos ombroclimas, como seco, subhúmedo, húmedo e hiper-húmedo, según la clasificación de Rivas-Martínez (1987). Según datos extraídos de una estación meteorológica situada en el valle del Guadalquivir (Fig. 1b) que ocupa una posición central respecto al perímetro del espacio protegido, esta zona tiene una precipitación anual media superior a los 900 mm, y la temperatura media anual se sitúa en torno a los 11ºC. La accidentada topografía, las

particularidades climáticas e históricas y la pobreza de sus suelos ya referidas han favorecido que estas sierras se encuentren en un relativo buen estado de conservación, razón por la cual se reconocen como Parque Natural en 1986, espacio protegido que comprende en la actualidad unos 2140 km². El tipo de bosques caracterizado por un estrato arbóreo dominado por los pinos y las encinas, acompañados por un matorral de sabinas, enebros y piornos, domina una amplia extensión de estas sierras (Luque, 1995). No obstante, y favorecidas por la diversidad de microclimas existentes en el territorio, son muchas las especies vegetales que se desarrollan en distintos nichos ecológicos. Así, se establecen las bases idóneas para el desarrollo de múltiples comunidades vegetales, hecho que, unido a activos procesos de especiación característicos de zonas aisladas, como las "islas montañosas", hacen que el Parque cuente con una gran riqueza de especies vegetales (Herrera, 1989; Benavente Navarro, 1996; Soriano, 1987). La flora del Parque contiene una importante representación de la flora europea, y a su vez también incluye un amplio número de endemismos (Blanca y Valle, 1986; Benavente Navarro, 1998).

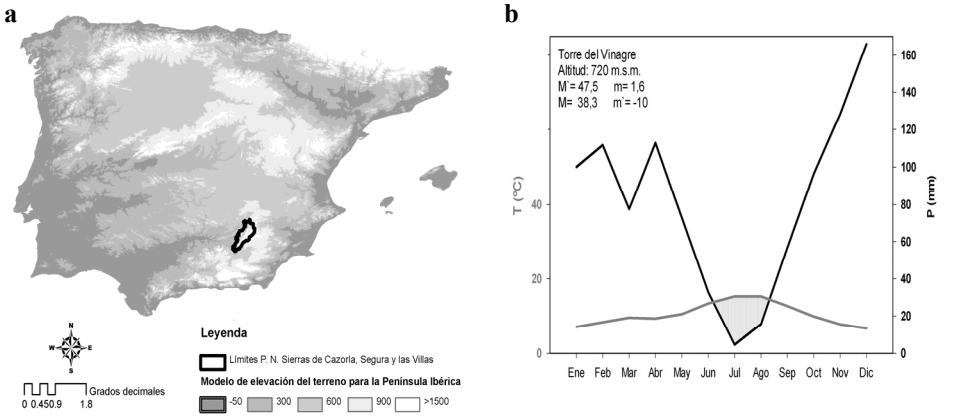


**Figura 1.** Localización y clima de la zona de estudio.
**a.** Mapa de la Península Ibérica señalando la ubicación del Parque Natural Sierras de Cazorla, Segura y las Villas, en el cual se realizó el trabajo de campo de esta tesis doctoral. Modelo digital del terreno disponible en http://www2.mncn.csic.es/LBI/relieve.htm.
**b.** Climatograma de la Estación Torre del Vinagre, en el P. N. Cazorla, Segura y las Villas. Se representa el promedio para un periodo total de 18 años de estudio (desde 1990) para las temperaturas medias mensuales (ºC, en gris), así como para la precipitación total mensual (mm, en negro), y en la esquina superior derecha se recogen la máxima absoluta (M') y media de las máximas (M) del mes más cálido (Julio), así como la mínima absoluta (m') y media de las mínimas del mes más frío (Diciembre).

Por todas las razones citadas anteriormente, esta región ha supuesto un espacio ideal para la realización de numerosos trabajos de investigación (ver selección de literatura sobre la historia natural del Parque en http://ebd06.ebd.csic.es/Publs.Cazorla.html), entre los cuales se encuentran los trabajos de recolección del néctar, análisis de las comunidades microbianas asociadas a insectos y plantas, determinación de riqueza específica, así como estudios de variables potencialmente relacionadas con los patrones de distribución de los microorganismos, centrados en distintas condiciones bióticas y abióticas, asociados a esta tesis doctoral.

Se estima que el 80% de las angiospermas de climas templados están polinizadas por animales (Ollerton *et al.*, 2011). Teniendo en cuenta estas estimas podemos calcular que el 7% de las presentes en el Parque fueron incluidas en el estudio de comunidades microbianas del néctar (Capítulo 1). Sin embargo, para la realización de aquellos capítulos de la tesis que precisaban de un diseño de muestreo más detallado se eligieron tres especies, como son el eléboro (*Helleborus foetidus*, Ranunculaceae), la dedalera negra (*Digitalis obscura*, Plantaginaceae) y el tabaco de pastor (*Atropa baetica*, Solanaceae). Las tres especies son autocompatibles (Herrera et al., 2001; Nebauer *et al.*, 1999; Herrera, 1987b); aunque presentan separación temporal de sus fases reproductivas masculina y femenina, es decir, poseen flores dicógamas (protoginas en el caso de eléboro y tabaco de pastor, y protándricas en el caso de la dedalera), mecanismo que favorece la polinización cruzada. Estas tres especies se suceden en floración a lo largo del año, y comparten polinizador principal, en este caso abejorros del género *Bombus* (Apidae). Los abejorros de este género suponen un importante polinizador en regiones templadas (Willmer, 2011) y en la zona de estudio se encuentran representados principalmente por tres especies, *B. terrestris*, *B. pratorum* y *B. pascuorum* (Castro, 1989). El eléboro (Lámina 1 a-c) es una planta de floración invernal, muy frecuente en el oeste de Europa. En la región de Cazorla podemos encontrar numerosas poblaciones de la especie, desde los 700 hasta los 1800 m.s.m. Posee flores grandes y muy duraderas (alrededor de dos semanas), que producen abundante néctar, recogido en varios

nectarios tubulares (una media de 5) situados en la base de la corola (Herrera *et al.*, 2001; Herrera *et al.*, 2002). La dedalera negra (Lámina 1 e,f) presenta distribución íbero-magrebí, donde crece en matorrales heliófilos, sobre sustrato calizo (Posadas Fernández, 2009). Es una variedad de dedalera perenne, que florece en los meses de Mayo-Junio. Sus flores, con una duración aproximada de seis días, producen cantidades crecientes de néctar a lo largo de su desarrollo, por lo que la fase de máxima producción coincide en este caso con la fase femenina. El tabaco de pastor (Lámina 1 h-j) es un endemismo ibero-marroquí que presenta graves problemas de conservación en la zona de estudio, donde se encuentran escasos y muy localizados núcleos de población de la especie, principalmente en zonas perturbadas y con influencia antrópica, desde los 1200 a los 1800 m.s.m. (Herrera, 1987b; Negrillo, 2009). Florece en verano, de Julio a Agosto, completando un proceso de crecimiento aéreo de los tallos que cubre un total de 5 meses del año (Blanca *et al.*, 1999). Las flores duran una media de 3-4 días, y producen cantidades moderadas de néctar en todas las fases de su desarrollo.

Estas tres especies, junto con sus polinizadores, han sido utilizadas como sistemas experimentales para estudiar el establecimiento y dinámica de las comunidades de levaduras del néctar. Las tres especies de planta presentan en sus tejidos compuestos secundarios con potencial actividad fungicida. El eléboro produce protoanemonina, una lactona insaturada para la que se ha descrito actividad fungicida (Mares, 1987). La dedalera negra produce cardenólidos como producto de su metabolismo secundario (Gavidia y Pérez, 1997), y el tabaco de pastor produce alcaloides tropanos (Adler, 2000; Zárate *et al.*, 2007). Solo los datos correspondientes a *Atropa baetica* y *Digitalis obscura* serán finalmente utilizados en esta tesis para el estudio de las comunidades de levaduras (Lámina 1 k,l), por ser su época de floración más similar, y responder así a unas condiciones naturales, abióticas y bióticas, más comparables. Además, sus compuestos del metabolismo secundario, a diferencia de la protoanemonina del eléboro, no presentan problemas de inestabilidad en su forma química, pudiendo incluso adquirirse comercialmente, lo que facilitó la parte práctica del trabajo.

**Lámina 1.** Localidades y especies que componen el sistema de estudio de esta tesis doctoral.

**a.** Detalle de la inflorescencia de *Helleborus foetidus,* parcialmente cubierta por nieve. Esta planta produce inflorescencias tras varias temporadas de crecimiento vegetativo, las cuales albergan generalmente entre 25 y 100 flores. **b**. Las flores de *H. foetidus* abren gradualmente a lo largo de su dilatado periodo de floración, que comprende desde Diciembre hasta Abril en nuestra zona de estudio. **c.** Coto del Valle, una de las seis poblaciones de *H. foetidus* estudiadas en el Capítulo 2 de tesis doctoral, situada a 960 m.s.m., bajo un bosque de pinos, quejigos y encinas. **d.** Puertollano, altiplanicie a 1800 m.s.m en la que domina un denso bosque de *Pinus nigra salzmannii*, y bajo el cual se ubica la población de *H. foetidus* situada a mayor altura de las incluidas en este trabajo. **e.** Flores campanuliformes de *Digitalis obscura,* dispuestas en inflorescencias unilaterales cuya antesis se produce secuencialmente, en sentido ascendente. **f.** Espigas alargadas de *D. obscura*, que pueden alcanzar hasta un metro de longitud, y que crecen principalmente en lugares de sotobosque aclarado. **g.** Valle de Guadahornillos, 1200 m.s.m., ejemplo de bosque mediterráneo bien conservado dentro del P.N. de Cazorla, Segura y las Villas. La vegetación dominante se compone de pino, encina, agracejo y madroño. **h.** *Bombus terrestris* succionando néctar de una flor de *Atropa baetica*. *Bombus* y *Psithyrus* son los principales visitantes de esta planta durante su floración. **i.** Detalle de una flor de *A. baetica*, en la que se observa los estambres ya maduros, aproximadamente a los 3 días de su apertura. **j.** Individuo de *A. baetica*, con los tallos ya elongados, en un momento previo a su floración. Esta especie se encuentra catalogada como "en peligro de extinción" según el libro rojo de la flora amenazada de Andalucía (Blanca *et al.*, 1999), por lo que la mayoría de los individuos presentes en el Parque se ubican dentro de cercados anti-herbivoría. **k.** Células de *Metschnikowia reukaufii* creciendo en néctar de *Helleborus foetidus*, fotografiada mediante microscopía de contraste de fase. **l.** Células de *Metschnikowia gruessii,* con su característica forma de crecimiento en forma de "aeroplano". Esta especie fue inicialmente confundida con la actual *M. reukaufii*, siendo ambas incluidas en tres géneros diferentes (*Anthomyces*, *Nectaromyces* y *Candida*,) hasta su descripción dentro del género *Metschnikowia* Kamieski (revisado en Lachance, 1998).

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**Yeasts inhabiting floral nectar: interspecific distribution patterns in Southern Spain**

# CAPÍTULO 1

*Levaduras en el néctar floral:*
*patrones interespecíficos de distribución en el Sur de España.*

# CAPÍTULO 1

## Yeasts inhabiting floral nectar: interspecific distribution patterns in Southern Spain

*Levaduras en el néctar floral: patrones interespecíficos de distribución en el Sur de España.*


## Abstract

While yeast presence in floral nectar has been known to science at least since the nineteenth century, it has more recently focused scientific attention from an ecological perspective. Under this new approach, nectarivorous yeasts have been related with a density-dependent degradation of nectar quality. However, quantitative information on the magnitude of occurrence of yeast contamination in floral nectar is scattered geographically and incomplete from the perspective of plant phylogenetic affiliation. Here, we document yeast frequency and abundance in nectar by examining microscopically nectar from 105 plant taxa for a single mountainous area in SE Spain. This 105 species corresponded to 25 families and 15 orders. Yeasts occurred regularly in the floral nectar of many species, as they were detected in the 37% of all nectar samples analysed microscopically. Nectar-living yeasts sometimes reached very high densities in individual nectar samples (up to $10^5$ cells/$mm^3$), although the averaged yeast abundance in nectar for the study area was $4x10^3$ cells/$mm^3$. Almost the 40% of the species surveyed contained yeasts, and there were significant differences in the magnitude of yeast colonization among plant species. The strongest component of variance in yeast abundance in nectar samples was caused by interspecific variation in the magnitude of yeast colonization, but significant patterns of variance in nectar yeast abundance also takes place at the intraspecific level. As a result, we were unable to find a clear phylogenetic signal in the described patterns of yeast incidence. We discuss the relevancy of these results in the context of evolution of the three-way interaction among Angiosperms, their pollinators and the nectarivorous yeasts.

## Resumen

La presencia de levaduras en el néctar floral ha recibido atención científica desde al menos, el siglo XIX, si bien su estudio desde una perspectiva ecológica es mucho más reciente. Algunos trabajos recientes han relacionando la abundancia de levaduras en el néctar con una degradación en la calidad de este recurso, pero aún son contados los estudios que presentan datos cuantitativos de la frecuencia y magnitud de contaminación del néctar floral de distintas especies de plantas. Concretamente, las estimas de incidencia de las levaduras en el néctar son incompletas desde un punto de filogenético, y los datos disponibles pertenecen a estudios en distintas regiones. Por ello, en este capítulo se examina el néctar de 105 especies, pertenecientes a 25 familias y 15 órdenes diferentes, para una única área de estudio, en este caso una zona montañosa del sureste de España. El 37% de las muestras de néctar analizadas microscópicamente albergaron levaduras. Estas levaduras alcanzaron, en ocasiones, valores de densidad muy elevados para una muestra de néctar, hasta $10^5$ cel/mm$^3$, si bien la abundancia promedio para nuestra área de estudio se situó en $4x10^3$ cel/mm$^3$. Se hallaron levaduras en casi el 40% de las especies cuyo néctar fue analizado. Se detectaron diferencias significativas entre especies en cuanto a la magnitud del fenómeno de colonización del néctar con levaduras. Sin embargo, aunque las diferencias interespecíficas explicaron el mayor porcentaje de la varianza existente para la abundancia de levaduras en muestras de néctar, las diferencias entre individuos de una misma especie también fueron altamente significativas. Por lo tanto, no pudo discernirse un patrón filogenético claro para la incidencia de levaduras descrita. La relevancia de estos resultados se discute en el contexto de la evolución tripartita entre las Angiospermas, sus polinizadores y las levaduras habitantes del néctar.


Datos parcialmente publicados en: Herrera, C. M., C. de Vega, A. Canto, and Pozo, M. I. 2009. Yeasts in floral nectar: a quantitative survey. *Annals of Botany* 103: 1415-1423.

## Introduction

Floral nectar, apart from its role as a reward that plants offer to their pollinators, usually shelter yeasts. Whereas the presence of yeasts in flowers and, more specifically, floral nectar, have long attracted the attention of microbiologists (reviewed in Herrera *et al.*, 2009), it has not been until the last decade that ecologists have studied possible effects of these microbes on plant-pollinator interactions. Recent investigations have revealed that yeast metabolism degrade floral nectar, namely by reducing total sugar concentration and by altering sugar and amino acid composition (Herrera *et al.*, 2008, Peay *et al.*, 2011) or even by causing nectary warming with respect to the floral surroundings (Herrera and Pozo, 2010). The magnitude of these effects was strongly dependent on yeast density, thus patchiness in yeast presence correspondingly generates patterns of variance in nectar quality (Herrera *et al.*, 2008; Pozo *et al.*, 2009). As a result, estimating the magnitude of yeast colonization in floral nectar has therefore become an aspect of great interest. However, studies assessing yeast frequency and abundance by examining microscopically nectar of individual flowers are still scarce.

Albeit yeasts are well known as ubiquitous organisms, either in terrestrial and aquatic environments (Kurtzman and Fell, 1998), several factors may ultimately affect the incidence of yeasts in floral nectar, as the colonization of this media imply some particular kind of difficulties. Dispersal dynamics conditions yeast frequency, as yeasts need vectors to colonize floral nectar, a spatially and temporally restricted media for microbial growth (Brysch-Herzberg, 2004; Canto *et al.*, 2008, Belisle *et al.*, 2011). Plant visitors are the main yeast dispersal agent between flowers, and a positive relationship has been found for certain types of plant visitors and nectar yeast incidence in several regions, i.e., bird-pollinated plants in South Africa, or bumblebee-pollinated plants in South Spain (Canto *et al.*, 2008; de Vega *et al.*, 2009). Interestingly, the relative composition of the flower-visiting comunity partially explains the interspecific differences occurring in the magnitude of yeast colonization (de Vega *et al.*, 2009; Herrera *et al.*, 2009) Moreover, temporal availability of flowers and nectar also restricts the opportunities for yeast

colonization. Although some environmental factors, along with the frequency of flower visits (Pleasants, 1983), effectively affect nectar volume, nectar availability is primarily determined by nectar secretory processes inherent to the plant itself (Pacini *et al*., 2003; Pacini and Nepi, 2007). Following yeast immigration to flowers, there are additional factors affecting yeast growth and survival, and thus, imposing limits to the abundance of these microbes in a given microhabitat (Fonseca and Inacio, 2006). Nectar-living yeasts are physiologically challenged by a sugar-rich aqueous solution with low concentrations of amino acids and other possible nitrogen sources (Baker and Baker, 1986; Nicolson and Thornburg, 2007). In addition, some plant species synthesize secondary compounds, which may entry in the nectary via phloem transportation (Adler, 2000; de la Barrera and Nobel, 2004). For some of these compounds, it have been demonstrated their antimicrobial function (Mares *et al*., 1987; Manson *et al*., 2007). Other plant species possess proteins in floral nectar, some of them specifically involved in defense against microbial pathogens (Carter and Thornburg, 2004).

Keeping in mind the restrictions that nectar traits impose to yeast growth noted above, and the genetic correlations among nectar traits and plant evolutionary history (e.g., Galetto and Bernardello, 2004; Mitchell, 2004), we thus hypothesize that nectar suitability to harbor yeast populations may be related to plant phylogeny. Generalizing the evidence of yeast presence along the Angiosperms is an essential step for a better understanding of the evolutionary significance of nectarivorous yeasts in the context of the plant-pollinator relationships. Thus, in this chapter we aim to assess quantitatively the presence of yeasts in the nectar of a large species sample scattered along the Angiosperms evolutionary tree, but growing in the same biogeographical region. Firstly, we will estimate, by means of microscopical examination of nectar samples, the frequency of occurrence (proportion of flowers whose nectar contained yeasts) and abundance (cell density in nectar) of yeast cells in the floral nectar of 105 plant species in a mountainous area in SE Spain. Secondly, we will focus on the

phylogenetic relatedness of the plant species surveyed, in order to explore the influence of plant species evolutionary history on nectar yeast incidence.

## Materials and Methods

This study was conducted at mixed pine-oak montane woodlands in the Cazorla, Segura y las Villas Natural Park, a very well preserved area in SE Spain. The study area is characterized by a cool Mediterranean climatological regime, with annual precipitation of 790 mm and mean annual temperature of 13ºC. Elevation ranged from 750–1600 m.

Floral nectar samples from 105 plant species, belonging to 25 families in15 orders, were examined microscopically for yeast cells. The taxonomic distribution among families of species sampled is shown in Table 1. Lamiaceae (24.8% of species), Asteraceae (7.6%), Fabaceae and Caprifoliaceae (6.7% of species each), along with Asparagaceae, Boraginaceae and Plantaginaceae (each contributing 5.7%), were the seven families contributing most species to our sample.

Flowering branches, inflorescences or single flowers of as many nectar-producing species as possible were collected from March 2008 through September 2010. The only criteria used for including a species in the survey were availability (at least 6-10 flowering, widely spaced individuals should be locally available, each bearing a minimum of 5-10 flowers) and that individual flowers generally produced measurable amounts of nectar (> 0.15 μL) within 12 h of collection.

Collected branches, inflorescences or flowers were kept in a portable cooler inside plastic bags or glass jars for a few hours after collection until taken indoors, and then kept at ambient temperature until extraction and microscopical examination of nectar samples, which was generally done within 12 h of collection. For every species included in the survey, separate nectar samples were obtained from each of 1-40 flowers (mean ± SD = 1.6 ± 0.5 flowers/plant) from each of 1-20 different individual plants (mean = 4.8 ± 1.5 plants/species) using calibrated microcapillaries. Between 6-65 nectar samples (mean = 21.8 ± 9.9 samples/species) were microscopically examined per species ($N$ = 2300 samples in total). Particular

care was taken to examine only nectar samples from flowers that were already open, and thus had been exposed to pollinator visitation, at the time of collection in the field.

The volume of nectar extracted from each flower (usually <1 μL) was estimated by the length of the column within a calibrated micropipette, and then diluted up to 5-8 μL by addition of 25-60% (depending on the observer's preferences) lactophenol cotton blue solution to facilitate microscopical examination. Yeast cell density (cells/$mm^3$ of nectar volume) was then estimated directly for each nectar sample under a microscope at 400x using a Neubauer chamber and standard cell counting methods. A rigorous identification of the microbes present in all nectar samples would have required culturing and isolation (e.g. Brysch-Herzberg, 2004), but this was impractical given the large number of yeast-containing samples examined in this study. We identified yeasts present in nectar samples from consideration of cell size and diagnostic morphological features such as, for example, presence of budding cells and large vacuoles containing highly refractive corpuscles (see e.g. Fig. 1 in Herrera *et al.*, 2008). This coarse level of taxonomic resolution was sufficient for the purposes of this study. For a small fraction of nectar samples we corroborated that the microbes present in nectar samples examined microscopically were yeasts as presumed. Nectar drops (1 μL) were streaked onto YM agar plates (1.0% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract, 2.0% agar) with 0.01% chloramphenicol, and incubated at 23ºC. Isolates were obtained from the resulting colonies, and partial sequences of the D1/D2 domain of the large subunit ribosomal DNA were obtained following methods in Kurtzman and Robnett (1998) and Lachance *et al.* (1999). Confirmation that the microbes involved were yeasts, as well as the identity of species involved, was obtained by BLAST-querying the GenBank database with the sequences obtained. Results of these identifications are reported as part of Chapter 3 of this thesis (Pozo *et al.*, 2009).

The phylogenetic tree depicting the evolutionary relationships among the 105 plant species involved in this study was constructed using the Phylomatic web

tool (http://www.phylodiversity.net/phylomatic/phylomatic.html; last accessed 10 November 2011). The resulting tree was then modified manually to incorporate information on yeast abundance data using TreeGraph (version 2.0).

**Table 1.** Plant species surveyed per Family and their contribution to total nectar samples analyzed for yeast presence.

| Family (Order) | Plant species, *N* | Proportion of the species surveyed (%) | Species name |
|---|---|---|---|
| Amaryllidaceae (Asparagales) | 5 | 4.8 | *Allium molly, Allium roseum, Narcissus cuatrecasasi, Narcissus hedraeanthus, Narcissus triandrus* |
| Araliaceae (Apiales) | 1 | 1.0 | *Hedera helix* |
| Asparagaceae (Asparagales) | 6 | 5.7 | *Anthericum liliago, Aphyllantes monspeliensis, Muscari comosum, Ornithogalum umbellatum, Polygonatum odoratum, Urginea maritima* |
| Asteraceae (Asterales) | 8 | 7.6 | *Carlina corymbosa, Carduus granatensis, Carduus tenuiflorus, Centaurea calcitrapa, Cirsium flavispina, Cirsium odontolepis, Cirsium vulgare, Ptilostemon hispanicus* |
| Boraginaceae | 6 | 5.7 | *Anchusa azurea, Cynoglossum creticum, Echium boissieri, Echium flavum, Echium vulgare, Lithodora fruticosa* |
| Brassicaceae (Brassicales) | 2 | 1.9 | *Erysimum myriophyllum, Sisymbrella aspera* |
| Caprifoliaceae (Dipsacales) | 7 | 6.7 | *Knautia arvensis, Lonicera arborea, Lonicera etrusca, Lonicera implexa, Lonicera periclymenum, Scabiosa andryalifolia, Valeriana tuberosa* |
| Caryophyllaceae (Caryophyllales) | 5 | 4.8 | *Saponaria ocymoides, Silene alba, Silene colorata, Silene lasiostyla, Silene vulgaris* |
| Convolvulaceae (Solanales) | 1 | 1.0 | *Convolvulus tricolor* |
| Cornaceae (Cornales) | 1 | 1.0 | *Cornus sanguinea* |
| Crassulaceae (Saxifragales) | 1 | 1.0 | *Pistorinia hispanica* |
| Ericaceae (Ericales) | 1 | 1.0 | *Arbutus unedo* |
| Fabaceae (Fabales) | 7 | 6.7 | *Anthyllis vulneraria, Astragalus incanus, Erinacea anthyllis, Psoralea bituminosa, Tetragonolobus maritimus, Vicia onobrychioides, Vicia villosa* |
| Iridaceae (Asparagales) | 4 | 3.8 | *Gladiolus illyricus, Iris foetidissima, Iris pseudacorus, Iris xiphium* |
| Lamiaceae (Lamiales) | 26 | 24.8 | *Acinos alpinus, Calamintha nepeta, Calamintha sylvatica, Cleonia lusitanica, Clinopodium vulgare, Lamium amplexicaule, Lavandula latifolia, Marrubium supinum, Nepeta tuberosa, Origanum virens, Phlomis herba-venti, Phlomis lychnitis, Prunella grandiflora, Prunella laciniata, Rosmarinus officinalis, Salvia blancoana, Salvia verbenaca, Satureja intricata, Sideritis incana, Stachys officinalis, Teucrium polium, Teucrium pseudochamaepitys, Teucrium rotundifolium, Teucrium webbianum, Thymus mastichina, Thymus orospedanus* |
| Lythraceae (Myrtales) | 1 | 1.0 | *Lythrum salicaria* |
| Oleaceae (Lamiales) | 1 | 1.0 | *Jasminum fruticans* |
| Orchidaceae (Asparagales) | 3 | 2.9 | *Dactylorhiza elata, Orchis coriophora, Platanthera algeriensis* |
| Orobanchacaae (Lamiales) | 1 | 1.0 | *Orobanche haenseleri* |
| Plantaginaceae (Lamiales) | 6 | 5.7 | *Anarrhinum laxiflorum, Antirrhinum australe, Digitalis obscura, Erinus alpinus, Linaria aeruginea, Linaria liliacina* |
| Polygalaceae (Fabales) | 1 | 1.0 | *Polygala boissieri* |
| Primulaceae (Ericales) | 3 | 2.9 | *Coris monpeliensis, Lysimachia ephemerum, Primula vulgaris* |
| Ranunculaceae (Ranunculales) | 4 | 3.8 | *Aquilegia cazorlensis, Aquilegia vulgaris, Delphinium nevadensis, Helleborus foetidus* |
| Solanaceae (Solanales) | 1 | 1.0 | *Atropa baetica* |
| Violaceae (Malpighiales) | 2 | 1.9 | *Viola cazorlensis, Viola odorata* |
| Xanthorrhoeaceae (Asparagales) | 1 | 1.0 | *Asphodelus albus* |

### *Statistical analyses*

All statistical analyses were conducted with the SAS package (SAS Institute, Cary, USA). Despite yeast abundance distribution was log-transformed for analyses, it was not well normalized in all cases, so we used non-parametric Kruskal-Wallis tests (NPAR1WAY Procedure) to estimate significance of differences in the mean log-transformed yeast abundance between plant species. We also tested the significance of differences in yeast frequency between plan species using the same procedure. Variance partitions and test of statistical significance of variance components in yeast abundance were estimated using restricted maximum likelihood (REML), as implemented in procedure MIXED, following a fully nested sampling scheme, with plant orders, families, genera, species, and individual plants belonging to the same species as the levels of variance examined, all of them were declared as random. The replicate counting on different flowers belonging to the same individual plant (see above) allowed us to assess the statistical significance of the among plants component of variation in yeast abundance in the majority of cases (95 out of 105 species, 378 observations out of 2300).

## Results

### *1. Yeast incidence*

In the Cazorla region and for the set of plant species surveyed, yeasts occurred quite frequently in floral nectar, as revealed by the high proportion of nectar samples that contained them 39.8% (*N*= 2300 flowers). When plant species rather than individual samples are treated as sampling units, mean (± SE; this notation will be used hereafter) per-species incidence of yeasts was 37.4 ± 3.4%, *N*= 105 species). There was, however, considerable interspecific variation in yeast frequency of occurrence ($\chi^2$= 539.2; d.f.= 104; p< 0.0001). The proportion of nectar samples containing yeasts encompassed the whole 0-100% range, and there was a continuum in yeast incidence running from species where yeasts were never recorded (30 species) to species where all nectar samples examined contained yeasts (4 species) (Fig. 1).

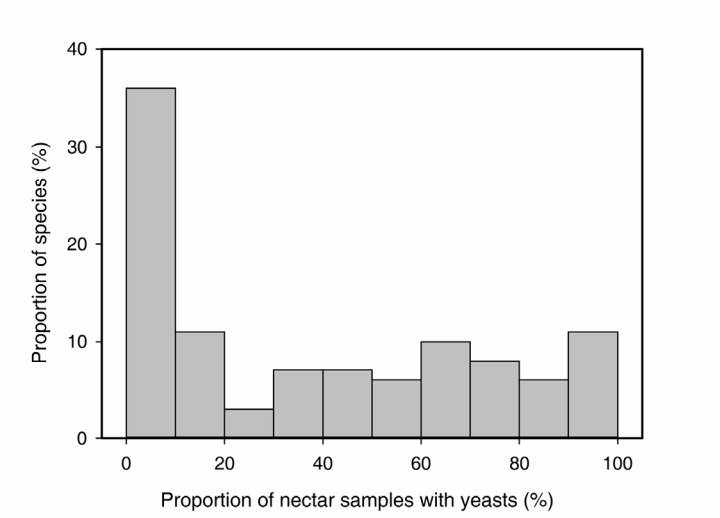



**Figure 1.** Frequency distributions of the proportion of nectar samples from a given plant species that contained yeasts.

Yeast cells reached extraordinarily high densities in some nectar samples, as denoted by the highest concentration recorded in a sample, $3{,}7x10^5$ cells/mm$^3$ (*Lonicera implexa*). Considering only samples with yeasts, the interquartile ranges of yeast abundance in individual nectar samples were $4{,}5x10^2$- $8{,}8x10^3$ cells/mm$^3$ (*N*= 915). When plant species rather than nectar samples were considered as units for analyses, the averaged value of species means of cell density was $4x10^3$ ($4{,}7 \pm 0.9x10^3$ Fig. 2, dashed line). Individual plant species strongly differed in mean yeast cell density estimates (Fig. 2).

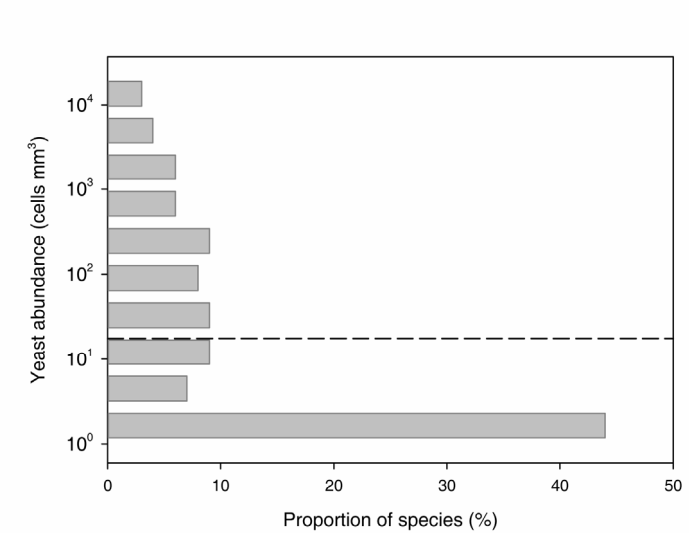



**Figure 2.** Frequency distributions of the mean yeast cell density in floral nectar samples of the 105 plant species surveyed. Dashed line denotes the average of species means. Samples with and without yeasts were included in the computations of means.

The range of variation of species means spanned nearly five orders of magnitude, and there were several species (16 of 105) with mean yeast cell densities above $10^4$ cells/mm$^3$(Table 2). Differences among plant species regarding mean yeast abundance were statistically significant ($\chi^2$= 561.6; d.f.= 104; $p<0.0001$).

**Table 2.** Frequency of nectar samples with yeasts (% samples with yeasts) and their averaged abundance (mean ± SE, cells/mm$^3$) for the 105 plant species surveyed.

| Plant species | Nectar samples, *N* | Yeast frequency | Yeast abundance |
|---|---|---|---|
| *Acinos alpinus* | 18 | 11.1 | 0.2 ± 0.1 x10$^3$ |
| *Allium molly* | 20 | 0 | 0 |
| *Allium roseum* | 14 | 14.3 | 0.6 ± 0.5 x10$^3$ |
| *Anarrhinum laxiflorum* | 18 | 94.4 | 0.6 ± 0.2 x10$^5$ |
| *Anchusa azurea* | 20 | 60.0 | 2,0 ± 0,7 x10$^2$ |
| *Anthericum liliago* | 18 | 0 | 0 |
| *Anthyllis vulneraria* | 23 | 43.5 | 1,1 ± 0,5 x10$^3$ |
| *Antirrhinum australe* | 18 | 77.8 | 1,8 ± 0,6 x10$^4$ |
| *Aphyllantes monspeliensis* | 18 | 0 | 0 |
| *Aquilegia cazorlensis* | 35 | 62.9 | 1,1 ± 0,6 x10$^4$ |
| *Aquilegia vulgaris* | 40 | 60.0 | 0.4 ± 0.1 x10$^4$ |
| *Arbutus unedo* | 30 | 0 | 0 |
| *Asphodelus albus* | 37 | 2.7 | 0.2 ± 0.2 x10$^0$ |
| *Astragalus incanus* | 19 | 0 | 0 |
| *Atropa baetica* | 60 | 30.0 | 0.4 ± 0.2 x10$^3$ |
| *Calamintha nepeta* | 24 | 91.7 | 3,3 ± 0.8 x10$^3$ |
| *Calamintha sylvatica* | 22 | 81.8 | 1,9 ± 0,5 x10$^3$ |
| *Carduus granatensis* | 27 | 18.5 | 0.3 ± 0.2 x10$^3$ |
| *Carduus tenuiflorus* | 16 | 0 | 0 |
| *Carlina corymbosa* | 19 | 57.9 | 0.7 ± 0.3 x10$^3$ |
| *Centaurea calcitrapa* | 23 | 0 | 0 |
| *Cirsium flavispina* | 25 | 24.0 | 0.6 ± 0.4 x10$^2$ |
| *Cirsium odontolepis* | 21 | 85.7 | 0.5 ± 0.2 x10$^4$ |
| *Cirsium vulgare* | 25 | 8.0 | 0.2 ± 0.2 x10$^2$ |
| *Cleonia lusitanica* | 20 | 0 | 0 |
| *Clinopodium vulgare* | 21 | 0 | 0 |
| *Convolvulus tricolor* | 20 | 0 | 0 |
| *Coris monpeliensis* | 13 | 69.2 | 2,1 ± 0,8 x10$^3$ |
| *Cornus sanguinea* | 19 | 0 | 0 |
| *Cynoglossum creticum* | 11 | 81.8 | 0.8 ± 0.2 x10$^4$ |
| *Dactylorhiza elata* | 23 | 0 | 0 |
| *Delphinium nevadensis* | 20 | 55.0 | 1,5 ± 0,5 x10$^3$ |
| *Digitalis obscura* | 44 | 90.9 | 3,4 ± 0,9 x10$^3$ |
| *Echium boissieri* | 18 | 0 | 0 |
| *Echium flavum* | 20 | 5.0 | 0.5 ± 0.5 x10$^1$ |
| *Echium vulgare* | 19 | 15.8 | 1,0 ± 0,7 x10$^2$ |
| *Erinacea anthyllis* | 20 | 40.0 | 0.3 ± 0.2 x10$^4$ |
| *Erinus alpinus* | 15 | 13.3 | 0.1 ± 0.1 x10$^3$ |
| *Erysimum myriophyllum* | 19 | 73.7 | 0.3 ± 0.1 x10$^5$ |
| *Gladiolus illyricus* | 33 | 69.7 | 0.5 ± 0.1 x10$^4$ |
| *Hedera helix* | 23 | 13.0 | 0.2 ± 0.1 x10$^4$ |
| *Helleborus foetidus* | 40 | 90.0 | 2,1 ± 0,6 x10$^4$ |
| *Iris foetidissima* | 17 | 76.5 | 0.6 ± 0.2 x10$^4$ |
| *Iris pseudacorus* | 16 | 100.0 | 0.3 ± 0.1 x10$^5$ |
| *Iris xiphium* | 16 | 6.3 | 0.5 ± 0.5 x10$^2$ |
| *Jasminum fruticans* | 19 | 47.4 | 0.6 ± 0.3 x10$^3$ |
| *Knautia arvensis* | 20 | 85.0 | 0.4 ± 0.1 x10$^5$ |
| *Lamium amplexicaule* | 19 | 0 | 0 |
| *Lavandula latifolia* | 23 | 13.0 | 0.2 ± 0.2 x10$^3$ |
| *Linaria aeruginea* | 20 | 20.0 | 0.4 ± 0.4 x10$^4$ |
| *Linaria lilacina* | 17 | 94.1 | 1,3 ± 0,4 x10$^4$ |
| *Lithodora fruticosa* | 19 | 73.7 | 1,6 ± 0,9 x10$^3$ |
| *Lonicera arborea* | 21 | 9.5 | 0,9 ± 0,7 x10$^1$ |
| *Lonicera etrusca* | 38 | 13.2 | 0.3 ± 0.2 x10$^3$ |
| *Lonicera implexa* | 22 | 36.4 | 0.2 ± 0.2 x10$^5$ |
| *Lonicera periclymenum* | 19 | 63.2 | 0.2 ± 0.1 x10$^4$ |
| *Lysimachia ephemerum* | 23 | 30.4 | 0.3 ± 0.2 x10$^3$ |
| *Lythrum salicaria* | 18 | 0 | 0 |
| *Marrubium supinum* | 18 | 88.9 | 0.6 ± 0.3 x10$^3$ |
| *Muscari comosum* | 18 | 33.3 | 0.4 ± 0.2 x10$^4$ |
| *Narcissus cuatrecasasi* | 65 | 69.2 | 0.6 ± 0.2 x10$^4$ |
| *Narcissus hedraeanthus* | 10 | 0 | 0 |
| *Narcissus triandrus* | 20 | 25.0 | 0.4 ± 0.3 x10$^4$ |
| *Nepeta tuberosa* | 22 | 90.9 | 1,1 ± 0,4 x10$^4$ |
| *Orchis coriophora* | 20 | 0 | 0 |
| *Origarum virens* | 21 | 76.2 | 0.3 ± 0.1 x10$^4$ |
| *Ornithogalum umbellatum* | 20 | 0 | 0 |
| *Orobanche haenseleri* | 28 | 17.9 | 0.5 ± 0.3 x10$^1$ |
| *Phlomis herba-venti* | 28 | 32.1 | 1,8 ± 0,9 x10$^2$ |
| *Phlomis lychnitis* | 20 | 45.0 | 1,4 ± 0,6 x10$^2$ |
| *Pistorinia hispanica* | 20 | 0 | 0 |
| *Platanthera algeriensis* | 20 | 0 | 0 |
| *Polygala boissieri* | 16 | 0 | 0 |
| *Polygonatum odoratum* | 14 | 64.3 | 0.6 ± 0.4 x10$^4$ |
| *Primula vulgaris* | 25 | 76.0 | 2,7 ± 0,9 x10$^4$ |
| *Prunella grandiflora* | 21 | 52.4 | 0.6 ± 0.3 x10$^3$ |
| *Prunella laciniata* | 20 | 0 | 0 |
| *Psoralea bituminosa* | 16 | 56.3 | 1,1 ± 0,4 x10$^4$ |
| *Ptilostemon hispanicus* | 24 | 75.0 | 2,5 ± 0,7 x10$^3$ |
| *Rosmarinus officinalis* | 29 | 72.4 | 0.5 ± 0.2 x10$^4$ |
| *Salvia blancoana* | 22 | 45.5 | 1,2 ± 0,5 x10$^2$ |
| *Salvia verbenaca* | 15 | 33.3 | 0.3 ± 0.2 x10$^4$ |
| *Saponaria ocymoides* | 8 | 0 | 0 |
| *Satureja intrincata* | 48 | 52.1 | 1,4 ± 0,8 x10$^3$ |
| *Scabiosa andryalifolia* | 21 | 85.7 | 2,1 ± 0,5 x10$^4$ |
| *Sideritis incana* | 26 | 100.0 | 2,7 ± 0,6 x10$^4$ |
| *Silene alba* | 15 | 0 | 0 |
| *Silene colorata* | 21 | 42.9 | 1,4 ± 0,6 x10$^3$ |
| *Silene lasiostyla* | 20 | 10.0 | 0,3 ± 0,2 x10$^1$ |
| *Silene vulgaris* | 13 | 0 | 0 |
| *Sisymbrella aspera* | 16 | 0 | 0 |
| *Stachys officinalis* | 22 | 31.8 | 0.3 ± 0.2 x10$^4$ |
| *Tetragonolobus maritimus* | 18 | 100.0 | 0.6 ± 0.2 x10$^4$ |
| *Teucrium polium* | 24 | 41.7 | 0.5 ± 0.3 x10$^3$ |
| *Teucrium pseudochamaepitys* | 20 | 95.0 | 3,4 ± 0,9 x10$^3$ |
| *Teucrium rotundifolium* | 28 | 17.9 | 0.5 ± 0.4 x10$^3$ |
| *Teucrium webbianum* | 21 | 66.7 | 1,5 ± 0.4 x10$^3$ |
| *Thymus mastichina* | 19 | 5.3 | 0.9 ± 0.9 x10$^1$ |
| *Thymus orospedanus* | 18 | 0 | 0 |
| *Urginea maritima* | 20 | 0 | 0 |
| *Valeriana tuberosa* | 6 | 0 | 0 |
| *Vicia onobrychioides* | 20 | 100.0 | 3,6 ± 0.9 x10$^4$ |
| *Vicia villosa* | 19 | 52.6 | 0.2 ± 0.1 x10$^4$ |
| *Viola cazorlensis* | 18 | 0 | 0 |
| *Viola odorata* | 12 | 66.7 | 0.2 ± 0.1 x10$^5$ |

***Variance among families and plant species***

Heavily yeast-loaded (mean cell density >2x10$^4$ cells/mm3) species are taxonomically quite diverse, belonging to a number of phylogenetically disparate families like, e.g., Plantaginaceae (*Anarrhinum*), Caprifoliaceae (*Knautia, Scabiosa*), Fabaceae (*Vicia*), Lamiaceae (*Sideritis*), Primulaceae (*Primula*), Brassicaceae (*Erysimum*), Iridaceae (*Iris*), Violaceae (*Viola*), or Ranunculaceae (*Helleborus*), which are widely scattered over the angiosperm phylogenetic tree (Fig. 3). In contrast, there were some plant families with nectar free or nearly free of yeasts, as Orchidaceae, Caryophyllaceae, or Boraginaceae.

Variation among orders, families, genera, species and individuals within species accounted altogether for the 66.4% of the total variance in yeast abundance in nectar samples. However, at this scale of sampling a high proportion of variance still remained unexplained by the model, and this component, which combined an error component and differences among flowers of the same plant, was also statistically significant ($Z$= 24.14, $p< 0.0001$; 33.6% of total variance). Most of the model-explained variance in the yeast abundance of nectar from individual flowers occurred between species within genera ($Z$= 3.3, $p= 0.0005$, 60.1% of the explained variance), followed by differences between individuals belonging to the same plant species ($Z$= 8.1, $p< 0.0001$, 19.6% of explained variance, Table 3).

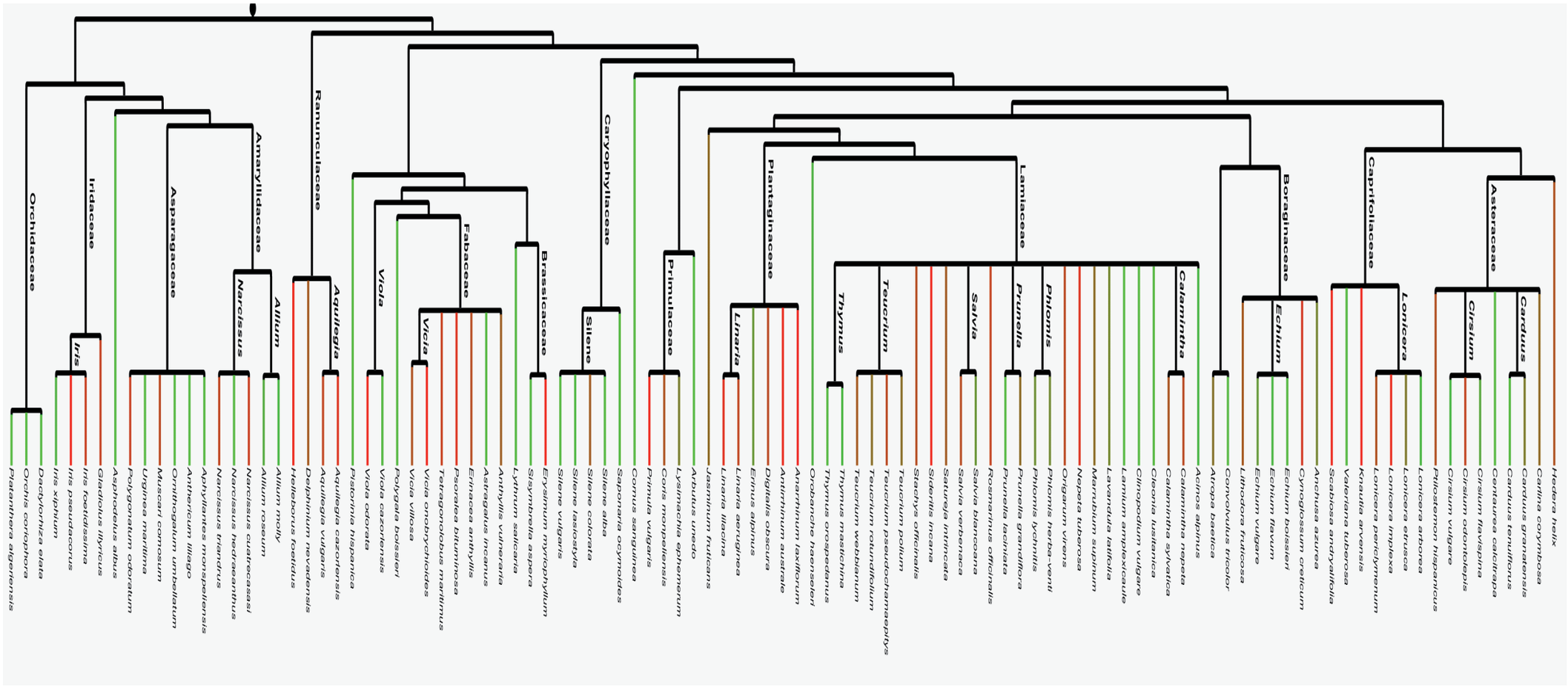
**Table 3.** Statistical significance of, and proportion of total variance due to, variation among orders, families, genera, species, and individual plants in the mean yeast abundance of 2300 nectar samples examined.

| Source of variation | Z | p | % of explained variance |
|---|---|---|---|
| Order | 0.06 | 0.4747 | 0.42 |
| Family (Order) | 0.70 | 0.2408 | 9.55 |
| Genus (Family) | 0.57 | 0.2849 | 10.37 |
| Species (Genus) | 3.32 | 0.0005 | 60.07 |
| Plant (Species) | 8.09 | <.0001 | 19.59 |

## Discussion

The main results of this study are that yeasts occurred regularly in the floral nectar samples from numerous plant species in the study region, and that they frequently reached high densities. Different plant species strongly differed in their yeast incidence estimates, so we were unable to disentangle clear patterns regarding the magnitude of yeast colonization at greater taxonomical scales, involving plant genera, families or orders. Our results serve to corroborate quantitatively the observations reported by previous studies and to extend them to a broader sample of plant species with disparate phylogenetic origin.

**Figure 3.** Phylogenetic relationships of the plant species sampled for this survey, and their estimated mean yeast abundance in nectar samples. Different colours in braches denote increments by 0.5 in log-transformed mean yeast abundance; departing from minimum (light green, 0), to intermediate (brown) and maximum yeast cell abundance (light red, 5).



The high frequencies of occurrence of yeasts in floral nectar found in our study are similar to those reported by the few previous microbiological investigations reporting comparable estimates of yeasts frequency in nectar samples. Jimbo (1926) assessed the frequency of occurrence of yeasts in floral nectar of 23 species from 14 families at several Japanese sites. For all species combined, the frequency of occurrence of yeasts in nectar samples (44%, *N* = 273 flowers) was close to the 39.4 % found in this study. For individual species, frequencies of occurrence in Jimbo's study ranged between 0-100%, and the mean (47.0%) was slightly higher to the figures found in this study for Cazorla (37.4%). For nine cultivated species in northwestern India, Sandhu and Waraich (1985) reported frequencies of occurrence of yeasts in floral nectar ranging between 39.5-92.1% (mean = 67.8%). Moreover, our yeast frequency estimates were remarkably similar to those obtained in nectar samples from South African forests and grasslands (43%, *N*= 635 flowers, de Vega *et al*., 2009) and lowland mediterranean scrub in southwestern Spain (32%, *N*= 740 flowers, Herrera *et al*., 2009). A higher frequency of samples with yeast, however, was obtained in deciduous tropical forests in Yucatán peninsula (54.4%; *N*= 675 flowers; Herrera *et al.* 2009).

Yeast density estimates obtained by directly counting cells under the microscope have been carried out less frequently. There is increasing quantitative evidence that yeasts can reach outstanding abundances at very distant and distinct ecological areas worldwide (Brysch-Herzberg, 2004; de Vega *et al*., 2009; Herrera*, et al*., 2009). These studies have also revealed that yeast cell densities in the order of $10^3$-$10^4$ cells/mm$^3$ are commonplace in nectar samples, and that densities >$10^5$ cells/mm$^3$ are not unusual (Herrera *et al.,* 2008; Herrera *et al*., 2009, de Vega *et al*., 2009). In this study we found an averaged value of species means of $10^3$ cells/mm$^3$, lower to those obtained in Yucatán and SW Iberian Peninsula ($10^4$; Herrera *et al*., 2009). In the present survey, maximum cell density per individual nectar samples was $4x10^5$, in agreement with the absolute ceiling of yeast abundance observed in SW Spain and Yucatán Peninsula (Herrera *et al*., 2009), but that is largely

superseded by some yeast counts in nectars of South African plants (de Vega *et al.*, 2009).

Quantitative surveys carried out on various plant species have revealed that there is a strong component of interspecific variance in nectar yeast abundance, and this work, that comprises the largest list of plant species for a unique study area, is not an exception. However, Herrera *et al.* (2009) and de Vega *et al.* (2009) examined the differences among species with respect to pollinator composition, and they found that those may explain the interspecific differences in the prevalence of nectar yeasts. In this work, we conducted a different approach, by examining if the taxonomical affiliation of species may account for a significant fraction of the wide-variance in nectar yeast incidence that takes place among plant species. Our nested variance estimates, considering differences among orders, families, genera, species and individuals of the same species, have shown that the largest component of variance in yeast abundance in floral nectar samples corresponded to variation among plant species within the same genus. Moreover, extensive variation also occurred within plant species, namely in this case among individual plants, a result that was previously pointed out by Herrera *et al.* (2008) for three plant species from our study area. Thus, we were unable to discern any general, broad-scale pattern of yeast distribution in nectar according to plant phylogeny. However, certain plant families like Fabaceae or Ranunculaceae where characterized by a high proportion of "heavily yeast-loaded" species, while others like Orchidaceae, Caryophyllaceae or Boraginaceae most often did not contain yeasts in nectar. Differences among plant families in yeast incidence were previously detected by Brysch-Herzberg (2004), although he was unable to find any clear relationship linking such variation with nectar properties like pH, sugar concentration, and sucrose/hexose ratio. Information on nectar characteristics is not available for the large list of species included in our survey, thus the role of variation in species- or family-specific nectar attributes in determining the size of yeast populations cannot be explored here.

Taking into consideration, as noted in the Introduction, that the magnitude of nectar degradation by microbes depended on cell density, our data also raise some important issues and implications for the study of the plant-pollinator relationships. Yeast abundances around $10^2$-$10^3$ cells/mm$^3$ have been found in the present survey for many of the plant species surveyed. Consequently, we might expect nectar degradation similar to that reported by Herrera *et al.* (2008) to occur frequently in many plant species in the field. The implications of this are two-fold. On the one hand, we should carefully control for the presence of nectarivorous microbes in floral nectar at the time of characterizing chemically field-collected nectar samples and ascribing those properties to intrinsic properties of plants. On the other hand, it opens new research directions with respect to the possible effects of yeast presence in plant-pollinator relationships, as yeasts seem to occur in a great proportion of the animal-pollinated species. In one hypothetical scenario, yeasts presence could impair plant fitness through their negative effects on pollinators. In such hypothetical situation, we may wonder how yeast presence in nectar could have been throughout the evolutionary history of Angiosperms, exemplified here by Asparagales as the oldest order and Dipsacales as the most recent one (Stevens, 2008). One possible explanation is that selective forces against yeasts could be unlikely to occur, as wide variance in yeast abundance occurred within plant species. Unfortunately, in this work we were unable to discern patterns of variance in yeast abundance at scales below the individual plant level. The study of nectarivorous yeasts and, in this particular case, their distribution patterns, apparently need to be "scaled –down" to focus on individual and subindividual variation, in order to evaluate more rigorously the unrecognized roles that nectar-living yeasts may play on plant pollinator behavior, and ultimately, on plant fitness.

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**What controls how many and where? Biotic and abiotic factors influencing distribution patterns of nectar yeasts in *Helleborus foetidus* flowers.**

# CAPÍTULO 2

*¿Qué controla dónde y cuántas? Factores bióticos y abióticos que afectan a los patrones de distribución de las levaduras del néctar en Helleborus foetidus.*

# CAPÍTULO 2

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*¿Qué controla dónde y cuántas? Factores bióticos y abióticos que afectan a los patrones de distribución de las levaduras del néctar en Helleborus foetidus.*


## Abstract

Yeasts are common inhabitants of floral nectar of insect-pollinated plants, where they can reach outstanding densities. Recent investigations have shown that nectar physical and chemical properties are modified by yeasts in a density-dependent way, dramatically decreasing its total sugar content, modifying sugar composition, or inducing a local warming inside the nectary with respect to the floral surroundings. However, distribution patterns of nectar yeasts in nature remain poorly investigated at multiple spatial and temporal scales. During two consecutive years (2008-2009) we measured incidence of nectar yeast in a single host plant, *Helleborus foetidus*, by quantifying their frequency and abundance across habitats (six populations located by pairs in an altitudinal gradient) and across flowering period (three collection dates for one population) in Southeastern Spain. Nectar yeast frequency and abundance varied widely across sites and dates. In addition, we focused on smaller scales of study, measuring how variance could be attributable at differences between individual plants, flower within plants and nectaries within flowers, obtaining that extreme variance occurs at he subindividual level. Keeping the present multi-scale mosaic of yeast abundance in mind, we explored several explanatory mechanisms potentially related to yeast contamination and proliferation in nectar samples, as pollinator visits, floral density and meteorological variables. Pollinator composition and activity were the main factors explaining the observed pattern of yeast incidence across nectar samplings. The extreme multi-scale variation in yeast incidence found in this study for *H. foetidus* may have also important consequences for the foraging activity of those incoming pollinators via nectar relationship, and ultimately on host plant reproductive success.

## Resumen

Las levaduras habitan frecuentemente en el néctar de las plantas polinizadas por insectos, en las cuales pueden alcanzar densidades muy altas. Investigaciones recientes han mostrado que las levaduras modifican, en función de su densidad, propiedades físicas y químicas del néctar, disminuyendo el contenido de azúcares totales, modificando la composición de azúcares, o bien induciendo un calentamiento del contenido del nectario respecto al aire circundante. Sin embargo, los patrones de distribución de las levaduras del néctar en condiciones naturales permanecen sin investigar a múltiples escalas espaciales y temporales. Durante dos años consecutivos (2008-2009) se midió la incidencia de levaduras en el néctar de una única planta hospedadora, *Helleborus foetidus*, cuantificando su frecuencia y abundancia en distintas localizaciones (seis poblaciones localizadas por pares a lo largo de un gradiente altitudinal) y a lo largo del periodo de floración (tres fechas de colecta para una misma población) en el sureste de España. La frecuencia y abundancia de levaduras en el néctar fue muy variable entre sitios y fechas. Además, se analizó la variación existente a escalas menores, como plantas individuales, flores dentro de plantas y nectarios dentro de flores, detectándose las diferencias más extremas dentro de los individuos. Se postulan diversos factores que podrían explicar el mosaico de variación descrito, como son variables meteorológicas, la densidad de flores, y la composición y abundancia de polinizadores, todas ellas relacionadas con la colonización y el crecimiento de las levaduras en el néctar. Estas variables fueron medidas durante el segundo año de muestreo, y al analizar su correlación con la incidencia de levaduras en los diversos muestreos se obtuvo que la composición de polinizadores es el factor más determinante para explicar el patrón observado. Un patrón de variación extrema en la incidencia de levaduras para el néctar de una única especie, como el que se describe en este capítulo, puede tener consecuencias muy importantes sobre la atracción y comportamiento de los polinizadores y el éxito reproductivo de la especie.


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## Introduction

Although the presence of yeasts in flowers, and more specifically, in nectar, has been reported repeatedly since the late nineteenth century by microbiologists and pollination biologists (see Herrera *et al.*, 2009 and references therein), some basic aspects about their ecology remain largely uninvestigated, including spatio-temporal distribution patterns. Yeasts may be common inhabitants of floral nectar, and yeast contamination rates of nectar samples above 60% had been recorded frequently for several regions scattered across the world (Jimbo, 1926; Sadhu and Waraich, 1985, de Vega *et al.*, 2009, Herrera *et al.*, 2009). Recent quantitative surveys of nectar yeasts suggested that yeast incidence in floral nectar can vary widely between regions, and host plant species within regions (Herrera *et al.*, 2009; de Vega *et al.,* 2009). In addition to interspecific differences in the prevalence of nectar yeasts, the nectar yeast incidence data provided by Herrera *et al.* (2009) and de Vega *et al.* (2009) revealed also considerable intraspecific variability (i.e., among individuals of the same plant species), but their sampling design, aimed to uncover broad scale patterns, did not explicitly address this issue.

As far as we know, there is no detailed study of variation in frequency and abundance of nectar yeasts, and their correlates, in a single plant species, an aspect that is essential to understand the ecological significance of yeasts as a third player in plant-pollinator interactions. In this paper, we present an analysis at yeast distribution patterns in the nectar of *H. foetidus* at various spatial and temporal scales. Several ecological and life history features render this plant species an ideal subject for studying yeast distribution patterns in floral nectar. Numerous aspects about the ecology of this plant specie are well known for our study site, and its floral nectar has been frequently associated with yeasts, as noted above. This herb is widely distributed in our study site, where it covers a wide elevational range, and its flowers open gradually over a long-lasting flowering period, thus enabling both spatial and temporal monitoring of nectar yeasts. The presence of several nectaries within each flower provides means for the analyses of the within-flower level when studying patterns of yeast occurrence.

We studied nectar yeast incidence during two consecutive years at six *H. foetidus* populations at different elevations in a mountainous area in SE Spain. Our main objective was to obtain quantitative measures of nectar yeast for the same host plant in different locations likely differing in some biotic (e.g., pollinator composition and activity, floral density) and abiotic features (e.g., air temperature, rain and air relative humidity) usually linked to changes in altitude in Mediterranean mountainous areas (Giménez Benavides *et al.*, 2006). We hypothesized that nectar yeasts local distribution patterns may be related to several environmental factors potentially influencing nectar availability, pollinator movement and yeast proliferation after colonization. In particular, regarding abiotic features, we considered air relative humidity because it affects nectar secretion rates and nectar concentration (Corbet *et al.*, 1979). Air temperature may affect some plant features as plant phenology, floral density (Sánchez-Lafuente, *et al.*, 2005), and nectar secretion and concentration (Freeman and Head, 1990). Temperature also affects pollinator composition and visitation rates (Herrera, 1995) and yeast growth and survival (Deak, 2006). Other weather variables, such as the frequency of precipitation events, can affect insect pollinators foraging (Herrera, 1995), which finally affects yeasts dispersal (Canto *et al.*, 2008). We also expected biotic variables as floral density to affect pollinator visitation rates. We will try to elucidate which sampling-specific factors (abiotic vs. abiotic) may better explain the observed mosaic of yeast incidence in nectar samples.

Finally, we focused on a single location for a temporal monitoring for nectar yeast along the flowering season, in parallel with the gradual changes in the weather conditions and pollinator activity. By measuring nectar yeast incidence in a spatially nested design, which extent to populations, individuals within populations, flowers within plants, and nectaries within flowers, we will study how variance is organized down to the population scale. Despite the limited spatial and temporal scope of this study, results provide new insights into yeast distribution patterns for a single host plant at a detailed scale. We have documented an extreme intraspecific

variation in nectar yeast incidence, a yeast patchy distribution that may be better explained by yeast dispersal patterns by different insect pollinators.

## Materials and Methods

### *Study species and sites*

*Helleborus foetidus* L. (Ranunculaceae) is a winter-blooming perennial herb widely distributed in Western Europe. Each plant produces one or a few inflorescences every year, and 20-75 flowers will open asynchronously trough the 1-3 months flowering season. Flowers are protandrous, and are primarily visited by bumblebees (Herrera *et al.*, 2001). Every individual flower lasts for 1-3 weeks, and usually bears five big horn-shaped nectaries deeply hidden inside a globose, pendant corolla. Each individual nectary may contain up to 5 μL of nectar (see Herrera*, et al.*, 2002, for further floral details).

This study was carried out during two consecutive flowering seasons at six *H. foetidus* populations growing in well preserved mountain forests at the Cazorla, Segura y las Villas Natural Park, Jaén province, SE Spain (see Herrera *et al.*, 2009, for further details of the study region). Populations were located by pairs along an altitudinal gradient, which roughly covers the elevational range of *H. foetidus* in our study area. The two low elevation sites were located at 960 and 1100 m.a.s.l., respectively ("L1" and "L2"), the two middle elevation sites ("M1" and "M2") were located at 1460 and 1540 m.a.s.l., and the two high elevation sites ("H1" and "H2") were at 1790 and 1810 m.a.s.l. Distance between populations at the same elevational level ranged from 0.8-2.5 km, and linear distances to populations at different elevational level ranged 2-10 km (Fig. S1).

Sites elevation, average abiotic conditions recorded (temperature, air relative humidity), pollinator composition and activity, and floral density are given in Table S1.

***Field and laboratory methods***

As peak bloom in each altitudinal level was delayed by approximately two weeks between low, mid and high elevation sites, we could not standardize collection date among elevations. As a result, we sampled simultaneously the two populations placed at the same elevation level, and we sequentially sampled the next elevation level over two weeks later. Collection dates were thus spaced enough to ensure enough flower availability, (> 2 flowers per plant). Considering that "L2" population has repeated measures of nectar yeast incidence, for spatial comparison purposes we selected the collection date that matches with the other population at similar altitude ("L1"). Flowering season tends to be shorter in high elevation sites, so we selected one of the lowest elevation sites ("L2") for a temporal monitoring of nectar yeast before and after the peak bloom.

Every year, ten randomly distributed *H. foetidus* plants bearing at least two open flowers were haphazardly chosen in each population at the beginning of the study. Flower age was standardized at the time of collection, and thus only middle-aged flowers were chosen, which coincided with intermediate floral sexual stage (at least one half of mature anthers). The 2008-2009 combined sample resulted in 240 nectaries examined for nectar yeasts (one nectary from each of the two flowers per plant, N=10 plants in each of the six populations, 2-year sampling). For the low elevation site "L2", the ten selected plants were sampled repeatedly on a bi-weekly basis, starting from the beginning of the flowering season. In 2008, 120 nectar samples were analyzed for nectar yeasts (two nectaries from each of two flowers per plant, and ten plants in each of the three collection dates from late February to late March. In 2009, warmer conditions resulted in a slightly longer flowering period at population level, so we monitored the population from early March to mid April and collected 132 nectar samples (2 nectaries from each of 2 flowers per plant, and $N$ = 10, 10, 9, and 4 plants, in four collection dates, respectively).

On each occasion, individually marked flowers were collected in the field, placed inside plastic containers and immediately moved to the lab, where they were kept in a refrigerator at 5ºC. In the lab, we then marked one ("L1", "M1", "M1",

"H1" , "H2") or two (temporal monitoring in "L2") nectaries per flower prior to nectar extraction, which was done as soon as possible and not later than 24 hours after field collection. We measured nectar volume using calibrated micro-capillaries and around one or two microliters were extracted from each nectary, dilluted in a 40% lactophenol cotton blue solution, and inmediatelly examined microscopically for yeast cell density determination. We conducted 16 replicated standard counts on each individual nectar samples using a Neubauer chamber, and a magnification of 400x. We assesses the presence of yeast in nectar by estimating yeast frequency (proportion of nectar samples with nectar yeasts) and abundance (mean number of cells $\mu l^{-1}$ of nectar). Microscopical examination of nectar indicated that all microbes involved in cell counts were unequivocally yeasts, and, more specifically, cell morphology resembled *Metschnikowia reukaufii*. Afterwards, identification was confirmed by growing aliquots of 1 µl of the individual nectar samples onto YGC agar plates (Yeast extract Glucose plus chloramphenicol agar, Fluka) to obtain individual colonies (*N*= 585 isolates), and by sequencing the D1/D2 domain of the large subunit of rDNA (*N*= 31 sequences), as described in Pozo *et al.*, (2011). All sequences were identified as *M. reukaufii* according to their high percentage of similarity (> 98%) with the type strain.

Temperature and air relative humidity records were obtained in each elevation level by placing a Gemini datalogger (Scientific house, Chichester, UK) held at the north face of a tree trunk, around at 3 m. of the ground. The dataloggers were programmed to record data every 30 minutes for a period of four months, between February and May 2009. We analyzed the following meteorological variables: mean, maximum, and minimum air temperature and mean relative humidity and the proportion of rainy days within the 15 days period before each collection date. We also analyzed the following three biotic factors, floral density pollinator composition, and pollinator activity. Floral density within each locality was estimated along a 70 m transect by using 7 consecutive circular areas of 10 m diameter in which we counted the number of plants, and the number of open flowers in each plant. Around 50 pollinator censuses of 3 min of duration were

conducted per site on two non consecutive sunny days around each collection date in the six *H. foetidus* populations to determine pollinator composition (% of flower visits accounted by one of the each two main visitors, *Apis* or *Bombus* sp.) and activity (flower visits/min) period following the methods described by (Herrera *et al.*, 2001; Herrera, 2005).

***Statistical analyses***

All statistical analyses were conducted with SAS package (SAS Institute, Cary, USA), unless otherwise explicitly specified. Yeast abundance distribution tended to be sharply bimodal reaching either extraordinary high values or falling on or near zero, thus this variable was log-transformed for analyses. Even so, yeast abundance distribution was not well normalized in all cases, and variance was heterogeneously distributed among groups, so we used non-parametric Kruskal-Wallis tests (NPAR1WAY Procedure) to estimate significance of differences in the mean log-transformed yeast abundance between elevations, populations and collection dates, analyzed separately by year. Variance partitions and test of statistical significance of variance components in yeast abundance were estimated using restricted maximum likelihood (REML), as implemented in procedure MIXED. Firstly, we carried out separate analyses of variance components for 2008 and 2009, following a fully nested sampling scheme, with populations, plants, and flowers as levels of variance examined, all of them were declared as random. The replicate counting obtained for each single-nectary sample allowed us to estimate measurement error and thus to assess the variance component and statistical significance of the among flower component of variation in yeast abundance. Within-population analyses were more accurately addressed for the "L2" population in which we measured two individual nectaries within flowers, allowing the variance partitioning between plants, flowers and nectaries as nested levels of variance, again all of them declared as random and with replicate countings included in the error term. The analyses were conducted for different years and collection dates separately.

We used a multivariate analysis of Partial Least Squares (PLS; SIMCA-P, v.12, *Umetrics* Inc.) to explore potential trade-offs between environmental features and yeast abundance. The rank transformed mean yeast abundance en each sampling (Wilcoxon mean scores, Conover and Iman, 1981), and the relative yeast frequency (% of nectar samples with yeasts) were the response variables. The explanatory variables included the biotic and abiotic parameters mentioned above. We then represented the contribution of each predictor in fitting the PLS model for both predictors and response, based on the *Variable Importance for Projection* (VIP) statistic as described in Wold *et al.* (2001), which summarizes the contribution a variable makes to the model. VIP values below 0.8 are considered to be not relevant enough for the VIP (Tenenhaus, 1998), so reference threshold has been placed at this value. For the purposes of PLS analyses, we considered the three different samplings in L2 as statistically independent units, given by the fact that different flowers were surveyed in each sampling.

## Results

### *1. Yeast incidence along an altitudinal gradient*

Frequency of nectar samples with yeasts varied widely among populations, and also between the two study years (Fig 1, left panels). In 2008, yeast frequency in nectar samples ranged from 0 (M2) to 90% (H2). In 2009, yeast frequency in nectar samples ranged from 0 ("L2") to 100% ("M1" and "M2"), and all the populations with presence of yeasts reached percentages of nectar samples consistently high (> 85%). We found that both nectar yeast incidence estimators for each population, frequency of nectar samples with yeast and mean yeast abundance per sample, were significantly correlated ($R^2$= 0.95; p< 0.0001; *N*= 12). In the following paragraphs we will analyze patterns of abundance in more detail.

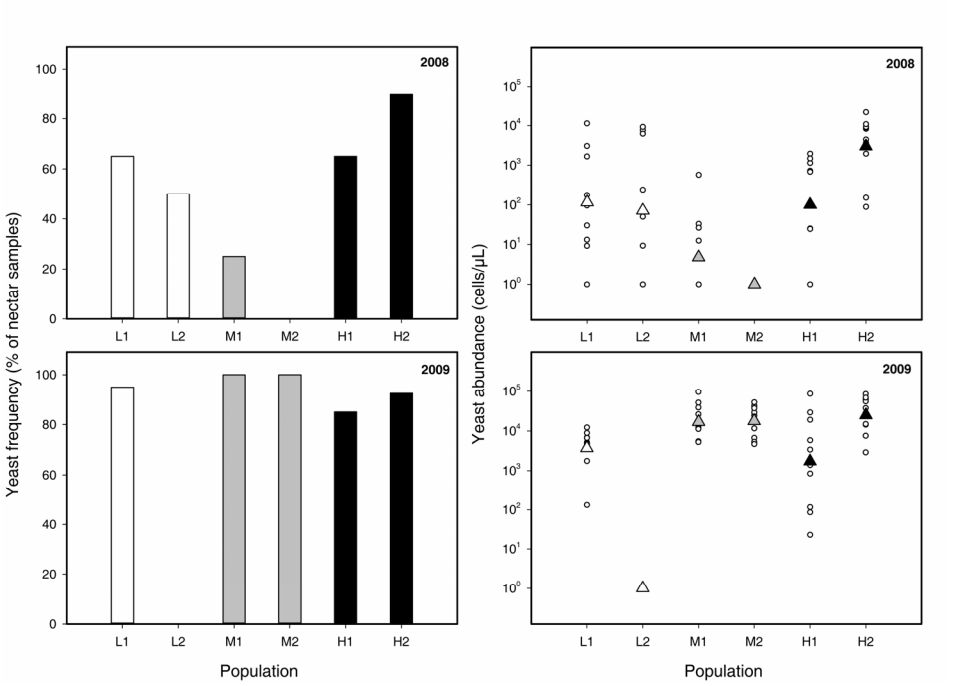


**Figure 1**. Yeast incidence in the six *H. foetidus* populations studied during two consecutive years, 2008 and 2009 up and bottom panels, respectively. Populations arranged by elevation, colours indicating the three elevation levels distinguished: low (L1, L2; white), middle (M1, M2, gray) and high (H1, H2, black). Right panels: yeast frequency (percentage of nectar samples with yeasts). Left panel: yeast abundance (number of cells $\mu l^{-1}$). Open circles denote mean cell density in each plant, and filled triangles mean cell density in each population.

Yeasts reached very high densities in some nectar samples. In 2008, the highest density in a sample was found in "H2", with near $4x10^4$ cells $\mu L^{-1}$, but three populations reached maximum yeast abundance values near to $2x10^5$ cells $\mu L^{-1}$ ("M1", "H1", and "H2") in 2009. Differences among populations in abundance of yeasts in floral nectar were statistically significant every year ($\chi^2$= 31.6 and 35.5, d.f.= 5, p< 0.0001, 2008 and 2009, respectively; Fig.1, right panels). Differences among populations remained statistically significant when only populations where yeast were recorded were compared (2008; M1 excluded, $\chi^2$ = 18.8, d.f= 4; p= 0.0008; 2009, L2 excluded, $\chi^2$= 15.5, d.f.= 4; p= 0.003). Within sites, nectar yeast abundance varied among individual plants (Fig. 1, right panels), but differences in

mean yeast abundance at the individual plant level were not statistically significant ($p > 0.12$).

Every year, differences among elevation categories in mean yeast abundance were statistically significant ($\chi^2 = 25.4$ and 23.1, 2008 and 2009, respectively; d.f= 2, $p < 0.0001$). Differences among populations did not follow a consistent altitudinal pattern (Fig. 1). High elevation sites were consistently characterized by high incidence of nectar yeasts. However, the middle elevation sites recorded the lowest and the highest, mean yeast incidence in 2008 and 2009, respectively.

***2. Seasonal variation***

Both frequency and abundance of nectar yeasts varied greatly among different collection dates along the flowering season in the single population studied (L2), and also showed a different pattern among the two consecutive years of nectar sampling (Fig. 2). Again, frequency and abundance were significantly correlated across collection dates ($R^2 = 0.96$; $p < 0.0001$; $N = 6$) and for the rest of the analyses we only analyzed abundance. In 2008, collection date effect on mean yeast abundance was statistically significant ($\chi^2 = 9.75$, d.f.= 2, $p = 0.007$). However, we did not observe the continuous variation expected according to gradual changes of meteorological conditions along the season, and yeasts were more frequent in nectar samples at the beginning of the blooming period in comparison with 'peak' and 'late' samples (Fig. 2).

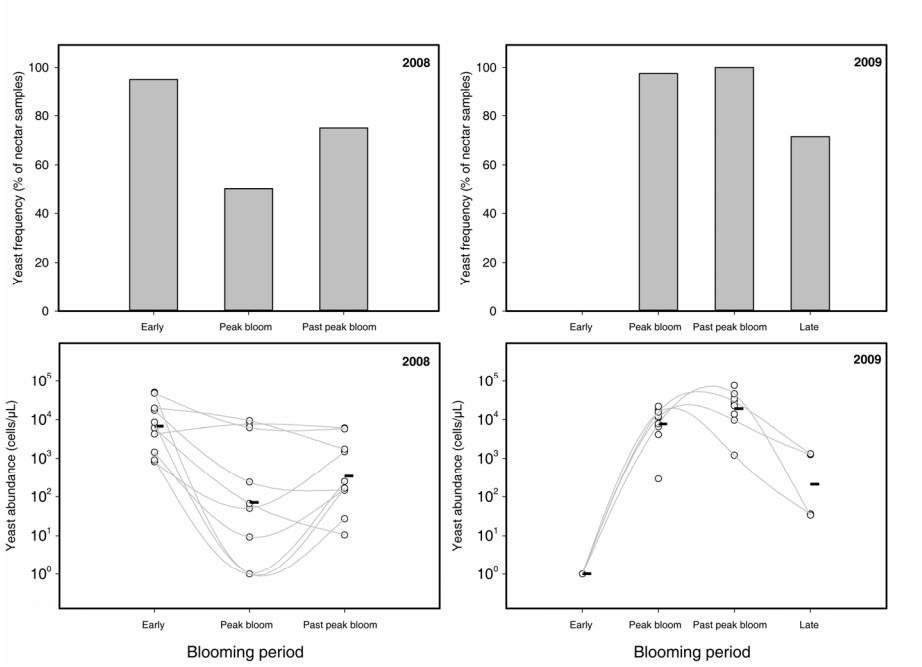


**Figure 2**. Yeast incidence in nectar samples according to plant phenology stage as followed in one *H. foetidus* population in 2008 and 2009, right and left panels, respectively. Upper panels: yeast frequency (percentage of nectar samples with yeasts). Bottom panels: mean yeast abundance (number of cells $\mu l^{-1}$). Open circles, individual plant means, and dark horizontal lines, collection date average. Curves connect the mean yeast abundance in individual plants blooming along the whole sampling period.

In 2009, yeast abundance showed a different pattern. Incidence initially increased and afterwards decreased, being yeasts most frequent and abundant at nectar samples at collection dates around the population peak bloom. Collection date effect on mean yeast density in nectar samples was statistically significant ($\chi^2$= 26.27, d.f.= 3, p< 0.0001), even if we just compare those collection dates in which yeasts were recorded ($\chi^2$= 10.68, d.f.= 2, p= 0.004). At the individual plant level, the same pattern emerged for those individuals that bloomed along the complete sampling period and yeast abundance increased along flowering period, reaching the maximum around population peak bloom and afterwards decreased (Fig. 2, curves in right panel). Differences among plant individuals in mean yeast abundance within each collection date were not statistically significant any year (p> 0.09).

### *3. Variation at nested spatial scales*

Variance component estimates provide a rigorous assessment of the relative importance of the different nested sources of variation considered, namely populations, plants and flowers. Differences between flowers of the same plant in yeast abundance were statistically significant ($Z$= 5.28 and 4.70, p< 0.0001, 37.1 and 39.7% of the total variance, in 2008 and 2009, respectively). Most of the model-explained variance in the yeast abundance of nectar from individual flowers occurred between flowers of the same plant, the smallest sampling level replicated in this analysis, being a consistent pattern between years (Fig 3).

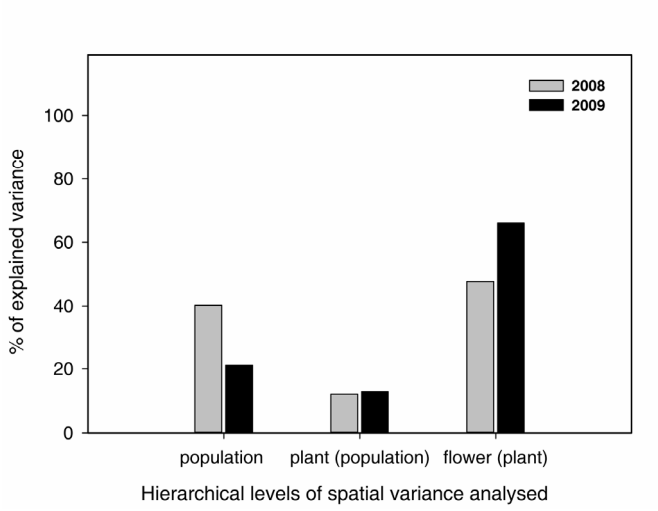


**Figure 3**. Dissection of variance components of yeast abundance in nectar samples of *H. foetidus* in a spatially nested scheme with population, plant within populations and flower within plants as the hierarchical levels of variance analyzed.

Moreover, at this scale of sampling a high proportion of variance still remained unexplained by the model and this component, that mixed the error and the within flower level, was also statistically significant ($Z$= 30 and 27, p< 0.0001; 22.3 and 39.7% of total variance, 2008 and 2009, respectively). As a result, we then focused on one population (L2), for which replicate measures for yeast abundance were also available for nectaries within flowers (Table 1). At collection dates corresponding with mid blooming stage, we obtained that a high percentage of the nectar-wide variance in yeast abundance was due to variation among nectaries of

the same flower, followed by variation among flowers of the same plant. However, Differences among nectaries of the same flower were statistically significant in the two years and at the two collection dates examined per year and accounted for >30% of the population-wide variance in all cases. However, differences among individual plants reached a higher relative importance at collection dates after the population peak bloom.

**Table 1.** Statistical significance of, and proportion of total variance due to: variation among plants, flowers within plants, and nectaries within flowers in the mean yeast abundance in single-nectary nectar samples in one population of *H. foetidus* (L2).

| | | 2008 | | | 2009 | | |
|---|---|---|---|---|---|---|---|
| Blooming period | Source of variation | Z | P | % of explained variance | Z | P | % of explained variance |
| Mid[a] | Plant | 0.84 | 0.19 | 20.69 | 0.81 | 0.21 | 19.80 |
| | Flower within plant | 0.85 | 0.19 | 21.03 | 0.59 | 0.28 | 15.40 |
| | Nectary within flower | 3.08 | **0.001** | 58.28 | 2.80 | **0.002** | 64.80 |
| Past peak bloom | Plant | 1.22 | 0.11 | 37.91 | 1.38 | 0.08 | 47.50 |
| | Flower within plant | 1.46 | 0.07 | 31.93 | 1.06 | 0.14 | 19.98 |
| | Nectary within flower | 3.04 | **0.001** | 30.16 | 2.70 | **0.003** | 32.52 |

[a.] It corresponded with "early" in 2008 and "peak bloom" in 2009, whereas no yeast where detected in the earliest sampling date of 2009.

### *4. Local environmental effects*

The relationships between yeast incidence in nectar samples and local abiotic and biotic parameters in each sampling occasion were explored by PLS analyses, in which the first three factors accounted for 75.1 % of the total variation in explanatory variables and for the 81.5 % of the total variation in response variables. According to the Variable Importance in Projection (VIP) scores plot (Fig 4b), the set of biotic variables had higher absolute coefficients and higher VIP than the set of abiotic variables. In particular, pollinator composition set of variables seemed to be the main mechanism underlying variance in yeast incidence, given by the fact that the proportion of flower visits by *Bombus* sp. and *Apis mellifera,* reached the highest VIP values (1.76 and 1.58, respectively), followed by their frequency of visits (0.85 and 0.90, respectively). According to the model coefficients, honeybee visits had a strong negative effect on both yeast abundance

and frequency in nectar samples (Fig 4a). In contrast, bumblebees mean abundance per census had uneven effects on yeast incidence. On one hand, a higher proportion of flower visits by bumblebees caused a positive effect on nectar yeast frequency, but on the other hand it caused a negative effect in yeast abundance. Floral density in the population did not have any effect on yeast incidence, and so, reached a very small value of VIP. Regarding abiotic predictors, only mean air temperature over the 15-d period preceding nectar sampling had a relevant effect on nectar yeast incidence (VIP value of 0.97), and this effect was mainly caused by a positive effect on nectar yeast frequency.

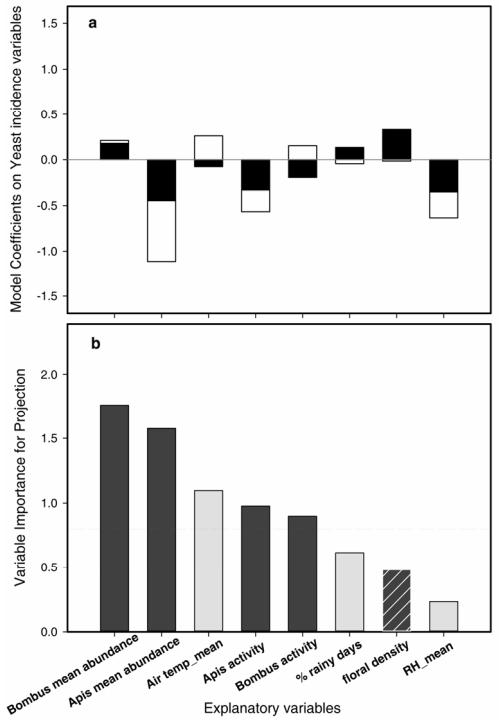


**Figure 4.** Variable importance plot.
(a) Model coefficients for explanatory variables in our three-latent factors PLS model (stacked bars, white for variable contribution on mean yeast frequency and black for contribution on mean yeast abundance). (b)Variable importance in projection (VIP) coefficients obtained from the PLS regression procedure. Explanatory variables ordered along the X-axis according to their explanatory power of Y (mean yeast frequency and rank transformed mean yeast abundance in nectar samples). Different colors used to indicate the type of variable: light gray for abiotic variables; and dark gray for pollinator composition and activity, and stripped bar for floral density).

## Discussion

The main results of this study are that the incidence of *M. reukaufii* in the floral nectar of one plant species can vary widely regionally and seasonally. Such variance was mainly accounted by differences at the intra-plant level, and thus did not produce consistent spatial and temporal patterns, associated to broader scales such as the altitudinal population gradient here investigated. Seasonal variation within a site was another important source of variation that pointed out to pollinators as the origin of the observed patchy distribution of this nectar-living yeast. The relevance of these results is discussed below.

### *Intraspecific variation of nectar yeasts*

The present study has documented wide intraspecific variability in the frequency of occurrence of yeasts inhabiting *H. foetidus* nectar. Even for a single study region and year, yeast frequency estimates ranged from 0 to 100%, depending on population and collection date. Two previous studies have estimated frequency of nectar yeast contamination in *H. foetidus*. In a German population, Brysch-Herzberg (2004) found that frequency of nectar samples with yeasts was 4% at the beginning of the blooming period and before the appearance of bumblebees, and 11% after first appearance of bumblebees; and the frequency of yeast-containing nectar samples ranged from 60% (female phase) to 100% at intermediate and male sexual stage (Herrera *et al.*, 2008) in 20 *H. foetidus* flowers colleted from one SE Spanish location. Moreover, the magnitude of intraspecific variation in nectar yeasts frequency found in the present study is similar, or even greater, than variation found in interspecific comparisons for the same study area (Herrera *et al.*, 2009). As a result, intraspecific variance should be taken into consideration when designing nectar yeast studies, since inaccurate assessment of nectar yeast presence in a given plant species may arise from inefficient sampling strategy.

### *Spatial variation of nectar yeasts*

Yeast incidence patterns can be readily discerned by either frequency of occurrence or mean yeast abundance data. Moreover, we carried out additional

statistical analyses on yeast cell counts, obtaining that collection date and site should be considered in studies dealing with nectar yeasts, as they both explained differences regarding yeast abundance in *H. foetidus* nectar. However, the contribution of differences among flowers of the same plant or even among nectaries of the same flower went beyond to the yeast abundance differentiation found at broader scales, thus causing inconsistent patterns at the population or the elevational level. The mosaic of yeast density occurring at the within-plant level, consistently obtained along the two years of sampling, has also important implications for the study of plant-pollinators relationships. On the one hand, we may expect wide-variation in nectar conditions (sugar composition and concentration, or nectar temperature) to occur at the within-plant level, as mediated by a density-dependent action of yeast metabolism (Herrera *et al.* 2006; Herrera *et al.,* 2008; Herrera and Pozo, 2010). Differences between contiguous nectaries in the same flower represent the smallest spatial scale perceived by foraging pollinators, so pollinator choice is likely to be affected by yeast patchy distribution across nectaries. On the other hand, the wide variance that takes place at the subindividual level decreases the probability of selective forces in plants to successfully act against nectar yeasts, in the case that those were effectively deterrent for pollinators.

***Local environment and nectar yeast incidence patterns***

Nectar wide-variance patterns at the within plant level are likely to be produced by factors operating at this scale, and, according to our initial expectations, we have corroborated that nectar yeast incidence patterns are ineluctably intertwined with yeast dispersal by certain types of pollinators. Our results corroborate the findings of Herrera *et al.* (2009) and de Vega *et al.* (2009), in which yeast incidence in nectar of several plant species was significantly related to pollinator composition. Moreover, although it was corroborated that bumblebees carried yeast effectively from one flower to another, it seems that not all pollinators are equally effective at vectoring yeasts to floral nectar, given by the fact that the nectar yeast frequency tend to decrease with increasing proportion of flower visits accounted by honeybees. Curiously, the experimental inoculation of unvisited *H.*

*foetidus* nectaries with honeybee glosses did not cause any characteristically "yeast-mediated" modification in nectar sugars (Canto *et al.*, 2008). Furthermore, the effect of bumblebee visits on nectar yeast abundance was frequency-dependent. Nectaries are initially sterile, so the frequency of visit of effective yeast-vectors, as bumblebees, is crucial to increase the frequency of yeast-contaminated nectar samples. However, if insect visits are not spaced farther apart, and period between visits is not long enough to permit cell proliferation inside the nectary to occur, we would detect a negative effect of visit frequency on nectar yeast abundance. In a winter blooming species such as *H. foetidus*, pollinators are scarcer, and every individual flower may be eventually visited once in a day (MIP, *pers. obs.*). Being the flower age equal, in this second scenario the few cells transferred in the initial colonization will be readily transformed into hundreds of cells inside the nectary, resulting in higher levels of nectar yeast abundance. Nevertheless, we stress the awareness on the implementation of cross-correlational analyses and analyses of factors, as used in this paper, as they may provide initial suggestions about the explanatory mechanisms for the studied season, but the reliability of these results need additional years of sampling, and their causality should be finally established by experimental manipulation (e.g., see Canto *et al.*, 2008).

***Seasonal variation of nectar-living yeasts***

Population blooming stage effectively affects yeast incidence in nectar samples, as it has been demonstrated by the yeast temporal monitoring in one *H. foetidus* population. Nectar yeast incidence varies along the flowering period, and it seems that nectar yeasts tend to be more frequent and abundant at collection dates around the peak bloom. This pattern was closely related to changing meteorological conditions along the flowering season, which in turns affected pollinator activity, but pollinator censuses and meteorological systematic records are simultaneously available only for the second year, unfortunately. For the 2009 season we may infer some consequences about the absolute lack of yeast for the first collection date, as *H. foetidus* is a winter-flowering herb, and one of the earliest blooming species in our study site. It is hypothesized that nectar-specialized yeasts, after passing an over

winter period that remains largely unknown, are indispensably transferred to nectar by emergent bee queens probing flowers in the early spring (Brysch-Herzberg, 2004, Canto *et al.*, 2008). In this context, our temporal monitoring of nectar yeasts, carried out simultaneously with pollinator censuses, gives support to these suggestions about the beginning of yeast annual cycle in floral nectar. Population phenology may help to explain the obtained altitudinal differences regarding nectar yeast incidence. Albeit we did not obtain a gradual change in nectar yeast incidence according to the altitudinal gradient, and the observed patterns were highly inconsistent among years, we consistently obtained that high elevation sites were characterized by high levels of yeast incidence in nectar samples. Plants growing at high elevation had a shorter blooming period that tends to compensate the flowering asynchrony that takes place in this host plant, so higher floral density at the population peak bloom usually occurs at high elevation sites. Increased floral density potentially affects pollinator activity (in fact, the highest elevation site, "H2", was that one with highest records of floral density and bumblebee abundance and activity), which in turn may raise the probability of yeast dispersal and proliferation inside the nectary.

Here we have reported the first systematic survey, to our knowledge, about the detailed quantification of nectar yeasts on a single host plant, comprising several spatial and temporal scales. The microscopical examination, along two years, of many nectar samples from different plants along different locations helps to provide a more comprehensive understanding of nectar yeast distribution patterns. The presented findings, taken altogether, may imply that nectar yeast presence intraspecific multi-scale mosaic even imposes, or overlies, a multi-scale variance in nectar features in a given plant species, that finally imposes unexplored connections with pollinators behavior and plant reproductive success. Considering that yeast presence have been proved to be a general feature along the angiosperms, the study of nectar yeast distributions patterns will help us move beyond anecdotal

observations to build a more predictive understanding of the role of nectar yeasts as "hidden-players" in the plant-insect pollinators ecological relationship.

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**Table S1**. Yeast incidence estimated as (a) and (b); and biotic and abiotic features based in individual values in each sampling for the 2009 *H. foetidus* flowering season. Averaged values are given in mean ± SD.

| | | | Yeast incidence | | Abiotic conditions | | | | Pollinators composition and activity | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Population | Blooming period | Elevation (m.a.s.l.) | Mean yeast abundance[a] (cells/µL) | Yeast frequency[b] (% of flowers with yeast) | Mean Relative humidity (%) | Frequency of rainy days (%) | Mean Air Temperature (ºC) | Mean floral density (N oper flowers/unit sample) | *Bombus* abundance (N/census) | *Bombus* activity (flowers/min) | *Apis* abundance (N/census) | *Apis* activity (flowers/min) |
| L2 | Early | 1100 | 0.00 ± 0.00 | 0.00 | 84.51 ± 18.23 | 46.67 | 5.84 ± 2.19 | 86.86 ± 77.11 | 0.26 | 7.13 | 0.17 | 2.82 |
| L2 | Peak | 1100 | 14611.64 ± 12344.03 | 97.50 | 71.99 ± 22.67 | 40.00 | 9.73 ± 1.47 | 136.69 ± 121.35 | 0.34 | 15.62 | 0.10 | 5.10 |
| L2 | Past peak | 1100 | 32923.02 ± 24942.47 | 100.00 | 72.81 ± 18.35 | 33.33 | 8.31 ± 3.17 | 96.70 ± 85.85 | 0.30 | 8.46 | 0.00 | 0.00 |
| L1 | Mid | 960 | 11096.40 ± 11444.87 | 95.00 | 87.72 ± 18.14 | 60.00 | 6.18 ± 2.47 | 36.84 ± 20.20 | 0.41 | 13.49 | 0.00 | 0.00 |
| M1 | Mid | 1460 | 39677.09 ± 52868.04 | 100.00 | 78.84 ± 13.46 | 46.67 | 5.58 ± 2.68 | 81.33 ± 34.38 | 0.16 | 5.95 | 0.00 | 0.00 |
| M2 | Mid | 1540 | 27855.33 ± 23734.49 | 100.00 | 78.84 ± 13.46 | 46.67 | 5.58 ± 2.68 | 30.04 ± 10.98 | 0.01 | 2.22 | 0.00 | 0.00 |
| H1 | Mid | 1790 | 22340.35 ± 42777.98 | 85.00 | 69.49 ± 22.19 | 53.33 | 7.68 ± 3.17 | 37.19 ± 45.15 | 0.36 | 12.84 | 0.04 | 2.86 |
| H2 | Mid | 1810 | 48194.68 ± 48029.68 | 92.50 | 69.49 ± 22.19 | 53.33 | 7.68 ± 3.17 | 279.36 ± 109.19 | 0.76 | 11.69 | 0.07 | 1.67 |

[a] Mean cell density (cells µl-1 of individual nectar sample) for each sampling. Samples with and without yeast were included for mean calculation.
[b] Proportion of nectar samples containing yeasts in each sampling.

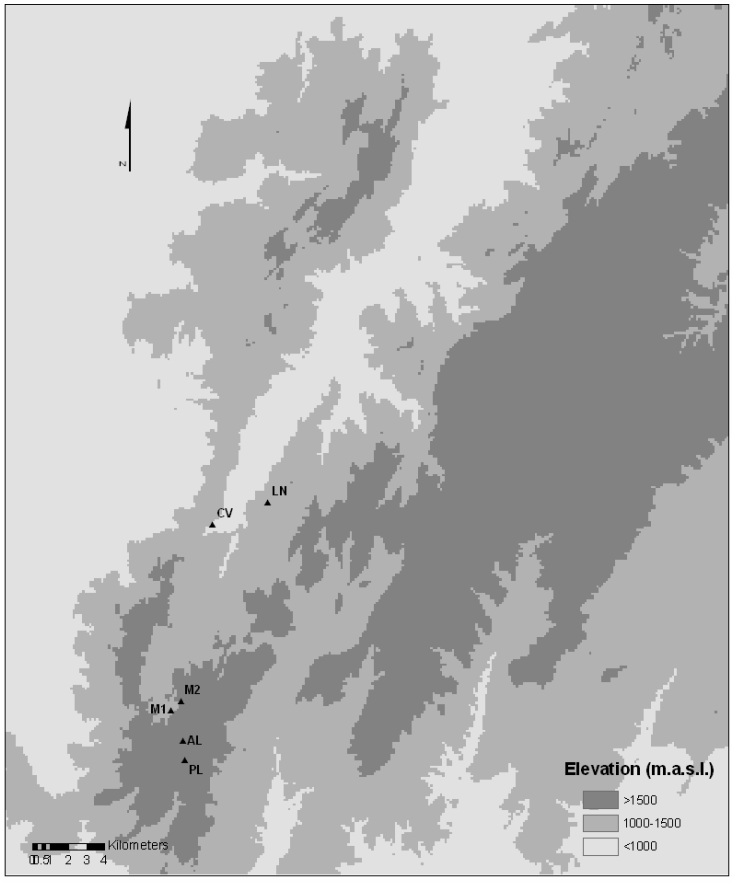
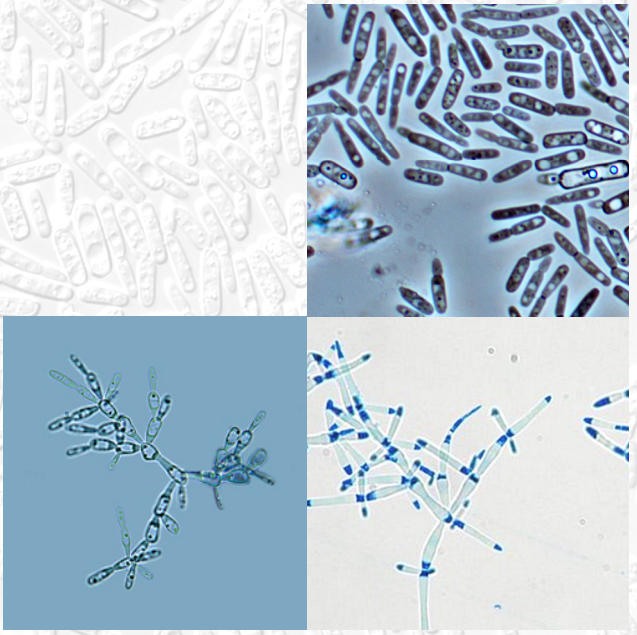



**Figure S1**. Spatial location of populations in the study area. Intervals of altitude (m.a.s.l.) are represented with grey scale (see legend).

**Species richness of yeast communities in floral nectar of southern Spanish plants.**



# CAPÍTULO 3

*Riqueza específica de las comunidades de levaduras del néctar floral en plantas del Sur de España.*

# CAPÍTULO 3

## Species richness of yeast communities in floral nectar of southern Spanish plants.

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## Abstract

Floral nectar of insect-pollinated plants often contains dense yeast populations, yet little quantitative information exists on patterns and magnitude of species richness of nectar-dwelling yeasts in natural plant communities. This study evaluates yeast species richness at both the plant community and plant species levels in a montane forest area in southern Spain, and also explores possible correlations between the incidence of different yeast species in nectar and their reported tolerance to high sugar concentrations, and between yeast diversity and pollinator composition. Yeast species occurring in a total of 128 field-collected nectar samples from 24 plant species were identified by sequencing the D1/D2 domain of the large subunit rDNA, and rarefaction-based analyses were used to estimate yeast species richness at the plant community and plant species levels, using nectar drops as elemental sampling units. Individual nectar samples were generally characterized by very low species richness (1.2 yeast species/sample, on average), with the ascomycetous *Metschnikowia reukaufii* and *M. gruessii* accounting altogether for 84.7% of the 216 isolates identified. Other yeasts recorded included species in the genera *Aureobasidium*, *Rhodotorula*, *Cryptococcus*, *Sporobolomyces* and *Lecythophora*. The shapes and slopes of observed richness accumulation curves were quite similar for the nectar drop and plant species approaches, but the two approaches yielded different expected richness estimates. Expected richness was higher for plant species-based than for nectar drop-based analyses, showing that the coverage of nectar yeast species occurring in the region would be improved by sampling additional host plant species. A significant correlation was found between incidence of yeast species in nectar and their reported ability to grow in a medium containing 50% glucose. Neither diversity nor incidence of yeasts was correlated with pollinator composition across plant species.

## Resumen

En las plantas polinizadas por insectos, el néctar floral contiene con frecuencia densas poblaciones de levaduras, si bien aún escasea la información sobre la riqueza específica de las comunidades que éstas constituyen. Este estudio evalúa la riqueza de especies tanto a nivel de la comunidad natural, formada por las flores individuales, como a nivel de especies de plantas, en una muestra representativa de localidades de bosque montano en el sur de España. Asimismo, se explora la existencia de correlaciones entre la incidencia de distintas especies de levaduras y su tolerancia a altas concentraciones de glucosa, y entre la riqueza de especies de levaduras que componen las comunidades a nivel de las distintas especies de planta y la composición de polinizadores. Las especies de levadura pertenecientes a 128 muestras de nectar, de 24 especies de planta diferentes, fueron identificadas por secuenciación del dominio D1/D2 de la subunidad 26s del rDNA. Se usaron análisis de rarefacción para estimar la riqueza de especies de levadura en el néctar de la comunidad de plantas y al nivel de las distintas especies, usando las gotas de néctar como unidades elementales del muestreo. Las distintas muestras de néctar se caracterizaron por una baja riqueza en especies (1.2 especies de levadura/muestra, en promedio), perteneciendo el 84.7% de las 216 colonias aisladas a los ascomicetos *Metschnikowia reukaufii* y *M. gruessii*. Otras especies encontradas se engloban en los géneros *Aureobasidium*, *Rhodotorula*, *Cryptococcus*, *Sporobolomyces* y *Lecythophora*. Las formas y las pendientes de las curvas de acumulación de especies observada fueron muy similares para los dos enfoques considerados, especies de planta y muestras individuales de néctar, pero ambos dieron lugar a distintas estimas de riqueza. Así, la riqueza estimada fue superior en el enfoque basado en las plantas hospedadoras, indicando que podrían aislarse especies de levadura adicionales en caso de ampliar el número de especies vegetales. La incidencia de una determinada especie en el néctar estuvo fuertemente correlacionada con su capacidad de crecimiento en medios con 50% glucosa. Sin embargo, no se halló una correlación significativa entre la incidencia de levaduras en el néctar, ni su diversidad, con la composición de polinizadores para las distintas especies de plantas estudiadas.

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## Introduction

The current best guess of planetary diversity at around 14 million species (Purvis and Hector, 2000) most likely represents a provisional underestimate, given our inability to survey exhaustively all sites and describe taxa before they disappear. Microorganisms contribute an important part to the as-yet-unknown biodiversity because of their vast densities and "misleading" diversity (Lambais *et al.*, 2006; Suh *et al.*, 2005). Molecular tools have dramatically expanded the range of microbial diversity, a significant part of which remained undetected with culture-dependent methods and morphological identification. Despite this, however, exhaustive inventories of microbial communities still remain largely impractical, and under most circumstances it is necessary to resort to sampling schemes in order to estimate their diversity (Hughes *et al.,* 2001). Techniques based on species accumulation curves and rarefaction (Colwell and Coddington, 1994;Gotelli and Colwell, 2001) have proven particularly useful to evaluate the relationship between number of species recovered and sampling effort, and could also be used to compare diversities of microbial communities for which samples of different sizes have been gathered. Microbial diversity differs widely across habitats. In marine microbial communities, for example, published estimates range from hundreds of Operational Taxonomic Units (OTUs) per milliliter in open water Schloss and Handelsman, 2005) to thousands in sediments Hong *et al.*, 2006. Apart from microbial surveys carried out in soil and aquatic environments, other terrestrial environments such as plant-associated microhabitats have been studied less frequently Colwell and Coddington, 1994,Qvit-Raz *et al.,* 2008). The diversity of yeast communities, and more specifically those that inhabit floral nectar, still remains a comparatively unexplored subsystem of the microbial communities associated with plants.

Although it has been long known that yeasts are frequent and can reach high densities in floral nectar (Brysch-Herzberg, 2004; Herrera *et al.*, 2009: and references therein), relatively little is known on their diversity levels and the factors that influence it Herrera *et al.* 2010). It may be hypothesized that nectar yeast

communities are shaped by the interplay between two conflicting forces. On one side, floral nectars, owing to its high energetic content Nicolson and Thornburg, 2007, could be considered highly favourable media for microbial growth. But on the other, their high osmotic pressure and frequent presence of secondary compounds (Adler, 2000; Manson *et al.*, 2007, Kyung *et al.* 2007) could frequently limit the number of yeast species that finally constitute nectar communities Herrera *et al.*, 2010, and references therein. In addition to its physical and chemical properties, floral nectar has another particularity. Yeasts need vectors to colonize floral nectar, hence insect visitation history and pollinator identity can be two additional factors influencing the diversity and composition of nectar yeast communities Canto *et al.*, 2008; Hong *et al.*, 2003; Lachance *et al.*, 2001).

The main objective of this study was to assess quantitatively the species richness of yeast communities in the floral nectar of a large sample of southern Spanish insect-pollinated plants. This study will also test if observed differences between yeast species in their frequency of occurrence in floral nectar are related to gross physiological differences, by relating the incidence of individual species with their reported tolerance to high osmolarity conditions. In addition, possible correlations across plant species between yeast species richness and pollinator composition will also be explored.

## Materials and Methods

### *Study site and methods*

This study was conducted during May-July 2008 in the Cazorla-Segura-Las Villas Natural Park in Jaén province, southeastern Spain, an area characterized by well-preserved pine-oak montane forests and woodlands (see Herrera *et al.*, 2009 0for additional information). At the time of this study, a large number of plant species were in bloom in the area and thus available for nectar yeast sampling. Additional data, including pollinator censuses and nectar yeast quantification for most plants included in this survey, were also available for the study region. A total of 128 nectar samples collected from flowers of 24 plant species belonging to 9

different families were cultured for yeast identification. A complete list of the species surveyed and their familial affiliations is shown in the Appendix. Fabaceae (21% of species), Lamiaceae, Plantaginaceae, Iridaceae (17% of species each), Caprifoliaceae and Ranunculaceae (8% of species each) were the families contributing most species to our sample. Brassicaceae, Oleaceae and Solanaceae each contributed a single species to the sample. The species studied here are a subset of those examined microscopically for occurrence of nectar-inhabiting yeasts in the same region by Herrera *et al.* (2009).

Flowering branches, inflorescences or individual flowers of study species were collected in the field and preserved in a portable cooler inside plastic bags or glass jars for a few hours until taken indoors, and then kept at ambient temperature. Extractions of 1 μl nectar samples, using calibrated microcapillaries, were conducted around 12-24 hours after field collection. Ten nectar drops per plant species were streaked individually onto Yeast Malt agar plates (1.0% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract, 2.0% agar) with 0.01% chloramphenicol, and incubated at 25ºC. Isolates were obtained from the resulting colonies following standard morphological criteria described in Yarrow (1998). The D1/D2 domain of the 26S subunit ribosomal DNA was two-way sequenced for all the isolates as described in 0 *et al.* (1999). Gblocks (Castresana, 2000) was used to trim the resulting alignment so that the beginning and ends of the consensus sequences were all the same. Although physiological characteristics are as important as molecular characterization for the identification of yeast species, for practical reasons we relied exclusively on DNA sequences for identification, which was accomplished by BLAST-querying the GenBank database (last accessed 17h December 2008). Although BLAST searches generally achieved very high similarity scores (usually between 90-100%, 91% of the sequences being above 98% similarity score), possible biases in richness estimates caused by the presence of undescribed species were also considered. To this end, this study evaluated operational taxonomic units (OTUs) defined on the basis of similarity of DNA sequences Hughes *et al.*, 2001, Lambais *et al.*, 2006, Shaw *et al.*, 2008). Determination of the

number of distinct molecular OTUs occurring in a set of DNA sequences, and assignment of sequences to OTUs, was done with the program DOTUR (Distance-Based OTU and Richness (Schloss and Handelsman, 2005). A PHYLIP (http://evolution.genetics.washington.edu/phylip.html) generated molecular distance matrix was used as input to DOTUR, which assigned sequences to OTUs based on a predetermined distance threshold. Pairs of isolates with molecular distances smaller than the chosen threshold were considered as belonging to the same OTU. A DNA dissimilarity cut-off of 3% was used in the analyses reported below. Although this value is larger than the 1% threshold suggested for species-level rDNA differentiation in yeasts (Kurtzman and Robnett, 1998; but see Lachance *et al.*, 2003), this value was chosen because it has been commonly used to distinguish "molecular" fungal species in environmental studies (Peay *et al.*, 2008), which would facilitate comparisons with other studies. In any case, the main conclusions of the DOTUR analyses were robust to variations in the OTU discrimination threshold in the range 1-3% (results not shown).

Statistical analyses

Densities of up to $10^5$ yeast cells/μl of nectar are often found in the wild (Herrera *et al.*, 2009), hence direct assessment of true yeast diversity in our samples was impractical and we had to rely on sampling (Hughes *et al.*, 2001). Sample-based rarefaction methods, applied to species presence-absence data, were used to assess overall yeast species richness at the plant community level (Colwell and Coddington, 1994) following procedures described by Colwell (2005) and Gotelli and Colwell (2001). Nectar drops will provide the elemental "samples" for all analyses, but two different approaches will be adopted to evaluate yeast species richness. In the first one, species occurrence data from all nectar samples will be analyzed together, irrespective of the plant species of origin. This procedure will provide "drop-based" rarefaction curves that assess overall species richness of nectar yeasts at the particular multispecific set of plant species which was sampled. Differences between plant species in life style or floral characteristics could influence their nectar yeast communities, so the second approach considered

separately the different host plant species from which nectar samples had been collected. In these analyses, data from all individual nectar samples from the same plant species were combined into a single sample and "plant species-based" rarefaction curves were obtained. Drop-based and species-based average rarefaction curves were computed with the EstimateS 8.0 program (Colwell, 2005), using 50 randomizations and sampling without replacement. Additionally, in order to estimate yeast species richness expected in nectar the nonparametric estimators ICE and Chao2 were used, because our taxa richness data are based on incidence (Chao *et al.,* 2005). Rarefaction generates the expected number of species in a small collection of n samples drawn at random from the large pool of N samples (Simberloff, 1978. In contrast, richness estimators predict the total richness of a community from samples. This study compared nonparametric estimators with the rarefaction analyses in order to evaluate the accuracy of richness estimates.

To test whether the frequency of occurrence of individual yeast species in floral nectar was related to their osmotolerance, yeasts species were classified into two categories according to their reported growth response in 50% glucose tests, namely "osmophilic" (positive growth response) and "non-osmophilic" (variable or negative growth response). Physiological data were obtained from Barnett *et al.* (2000) and the on-line CBS Yeast database available at http://www.cbs.knaw.nl/yeast/BioloMICS.aspx, except for *Aureobasidium pullulan*s and *Metschnikowia* unknown sp., for which we determined experimentally their responses to variable glucose concentration. Frequencies of occurrence of osmophilic vs. nonosmophilic species in both nectar drops and plant species were compared with the Wilcoxon rank-sum test, using the NPAR1WAY procedure in SAS and exact *P*-value estimation (SAS Institute, Cary, NC, USA).

Correlations across plant species between pollinator composition and yeast incidence were explored using detailed data on pollinator composition obtained in the study region for 12 of the 24 plant species sampled (Herrera et al, 2009; and C.M. Herrera, unpublished data). Only for this analysis we refer to "diversity" instead of "species richness", as both species richness and relative species

abundances are incorporated into the Shannon-Wiener diversity indices. A Principal Components Analysis was conducted on the variance-covariance matrix of the proportion of flower visits contributed by bumble bees, solitary bees, Lepidoptera, Coleoptera and Diptera in order to reduce the number of variables describing pollinator composition of each plant species. The fist component, which was mainly correlated with the frequency of flower visits by bumblebees and solitary bees (correlations = 0.6 and 0.4, respectively), accounted for the 73.2% of total variance. Relationships between nectar yeast incidence and diversity, and pollinator composition, were explored by correlating the frequency of occurrence, yeast abundance and yeast diversity (Shannon-Wiener index) of yeast cells in nectar, on one side, with the pollinator composition in each plant represented by scores on the first PCA component, on the other. The yeast-related variables mentioned above are also correlated with pollinator diversity calculated by Shannon-Wiener index. For statistical analyses, nonparametric tests were applied (Spearman rank correlation) as implemented in SAS 9.1 statistical package (SAS Institute, Cary, NC, USA) CORR procedure.

## Results

### *Observed species richness*

A total of 128 nectar drops were streaked onto culture media and the resulting 216 yeast isolates yielded a total of 12 species, comprising both ascomycetous and basidiomycetous yeasts (Table 1). The first group included *Metschnikowia reukaufii*, *M. gruessii* and an unidentified *Metschnikowia*, along with the "black yeasts" *Aureobasidium pullulans* and *Lecythophora hoffmannii*, while the second group included species of *Rhodotorula*, *Cryptococcus* and *Sporobolomyces*. Nectar communities sampled had very low species richness, since on average (± SE) only 1.3 ± 0.6 yeast species were recovered from each nectar drop. The two dominant species, *M. reukaufii* and *M. gruessii*, were recorded in 73.4% and 29.7% of nectar drops, respectively.

**Table 1.** Plant species from which nectar yeast isolates were achieved, number of DNA isolates, number of DNA isolates containing different yeast taxa.

| Plant species | DNA sequences, *N* | *Metschnikowia reukaufii*, *N* | *Metschnikowia gruessii*, *N* | *Aureobasidium pullulans*, *N* | *Cryptococcus* spp. [a] *N* | *Rhodotorula* spp. [b] *N* | *Sporobolomyces roseus*, *N* | *Lecythophora hoffmannii*, *N* | *Metschnikowia* sp., *N* |
|---|---|---|---|---|---|---|---|---|---|
| *Anthyllis vulneraria* | 19 | 17 | | 2 | | | | | |
| *Antirrhinum australe* | 9 | 5 | 2 | 2 | | | | | |
| *Aquilegia vulgaris* | 13 | 13 | | | | | | | |
| *Aquilegia cazorlensis* | 7 | 7 | | | | | | | |
| *Atropa baetica* | 20 | 3 | 14 | | 1 | 2 | | | |
| *Digitalis obscura* | 26 | 2 | 11 | 4 | | 7 | 1 | | 1 |
| *Erysimum mirtophyllum* | 1 | 1 | | | | | | | |
| Erinacea anthyllis | 12 | 8 | | 2 | 1 | | 1 | | |
| *Gladiolus illyricus* | 7 | 3 | 4 | | | | | | |
| *Iris foetidissima* | 14 | 14 | | | | | | | |
| *Iris pseudacorus* | 2 | 2 | | | | | | | |
| *Iris xyphium* | 2 | | | | 2 | | | | |
| *Jasminum fruticans* | 2 | | 2 | | | | | | |
| *Linaria aeruginea* | 9 | 9 | | | | | | | |
| *Linaria lilacina* | 14 | 14 | | | | | | | |
| *Lonicera etrusca* | 2 | 2 | | | | | | | |
| *Lonicera implexa* | 11 | 9 | 1 | | | | | 1 | |
| *Marrubium supinum* | 10 | 9 | 1 | | | | | | |
| *Phlomis lychnitis* | 6 | | 6 | | | | | | |
| *Prunella grandiflora* | 7 | 3 | 4 | | | | | | |
| *Tetragonolobus maritimus* | 13 | 13 | | | | | | | |
| *Teucrium rosmarinacaeyrtis* | 4 | | 4 | | | | | | |
| *Vicia onobrychioides* | 3 | 3 | | | | | | | |
| *Vicia villosa* | 3 | 3 | | | | | | | |

[a] *Cryptococcus* spp. includes *C. aerius*, *C. diffluens*, *C. uzbekistanensis* and *C. victoriae*.
[b] *Rhodotorula* spp. includes *Rh. colostri* and *Rh. mucilaginosa*.

The rest of species were much less frequent, and included *A. pullulans* (found in 7% of nectar samples), R*hodotorula colostri* (3.9%), R. *mucilaginosa* (1.6%), and *Sporobolomyces roseus* (1.6%). Species that were recorded only once (<1% of nectar samples) included *Cryptococcus aerius, C. diffluens, C. victoriae*, *C. uzbekistanensis*, *L. hoffmannii* and the unidentified *Metschnikowia* sp (Fig. 1). DOTUR analysis of the DNA sequence data for these isolates provided a slightly higher estimate of total species richness, as a total of 18 OTUs were identified at the 3% DNA dissimilarity

cut-off. The possibility that additional undescribed species were present in our samples cannot therefore be ruled out.

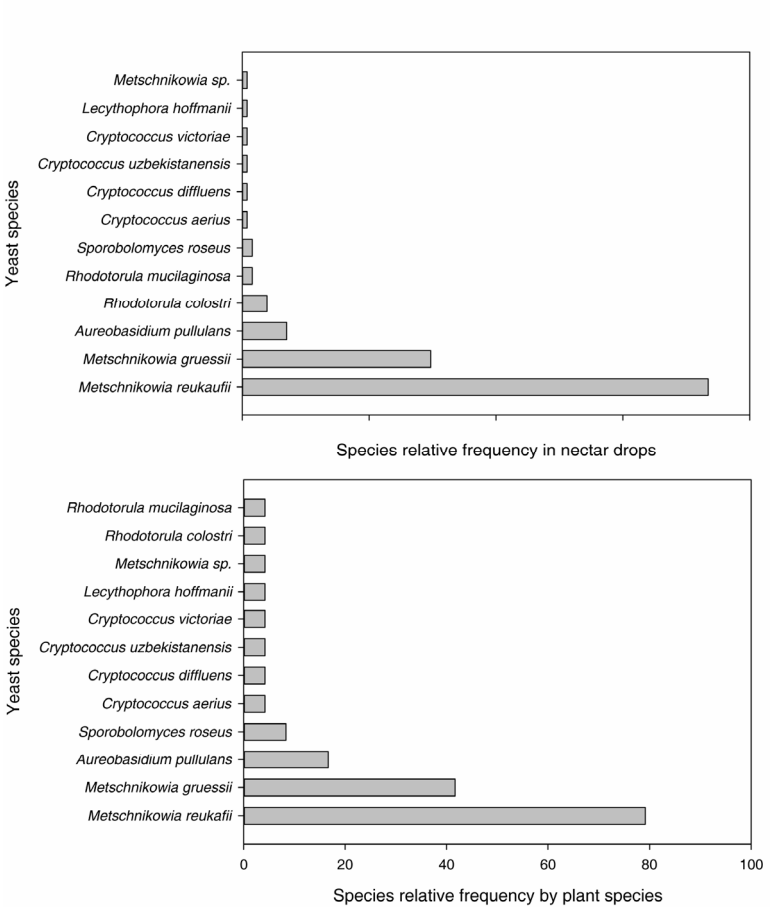


**Figure 1**. Yeast and yeast-like growing species frequency in nectar samples: drop-based (upper panel) and plant species-based (lower panel) approaches.

When plant species rather than nectar drops were considered as the samples for analyses, the pattern of yeast community species richness was similar: *Metschnikowia reukaufii* and *M. gruessii* were isolated from 20 and 10, respectively, of the 24 plant species surveyed. The rest of species occurred in a much reduced subset of plant species. *Aureobasidium pullulans* and *S. roseus* were found in 4 and 2 plant species, respectively, while the other species occurred in a single plant species each. The most species-rich yeast community occurred in the nectar of *Digitalis*

*obscura* (6 species, Table 2), followed by *Erinacea anthyllis* and *Atropa baetica*, with four yeast species each. *Antirhinum australe* and *Lonicera implexa* harboured three species each, and two species were recovered from the nectar of *Gladiolus illyricus*, *Prunella grandiflora, Anthyllis vulneraria* and *Marrubium supinum.* Only one yeast species occurred in the nectar of each of the remaining species.

**Table 2**.Yeast incidence and species richness, along with pollinator composition for the 12 Cazorla species included in the analysis of the relationship between pollination mode and presence of yeasts.

| Plant species | Yeast frequency (% samples with yeasts) | Yeast density (cells/mm$^3$) | Yeast diversity (H`) | Pollinator diversity (H`) | Pollinator composition (% flower visits) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Bumblebees | Solitary bees | Lepidoptera | Coleoptera | Diptera |
| *Anthyllis vulneraria* | 43.5 | 1141 | 0.15 | 0 | 0.0 | 100.0 | 0.0 | 0.0 | 0.0 |
| *Aquilegia cazorlensis* | 62.9 | 11127 | 0.00 | 0.29 | 77.0 | 46.0 | 18.4 | 0.0 | 0.0 |
| *Aquilegia vulgaris* | 60.0 | 4356 | 0.00 | 0.14 | 89.7 | 10.3 | 0.0 | 0.0 | 0.0 |
| *Atropa baetica* | 17.0 | 699.0 | 0.40 | 0.29 | 59.5 | 40.5 | 0.0 | 0.0 | 0.0 |
| *Digitalis obscura* | 90.9 | 3402 | 0.63 | 0.09 | 94.0 | 6.0 | 0.0 | 0.0 | 0.0 |
| *Erinacea anthyllis* | 40.0 | 2948 | 0.43 | 0.37 | 33.5 | 59.3 | 7.2 | 0.0 | 0.0 |
| *Gladiolus illyricus* | 69.7 | 5048 | 0.30 | 0.30 | 0.0 | 65.0 | 33.8 | 0.0 | 1.2 |
| *Linaria aeruginea* | 20.0 | 4062 | 0.00 | 0.23 | 0.0 | 76.9 | 0.0 | 23.1 | 0.0 |
| *Lonicera etrusca* | 13.2 | 316 | 0.00 | 0.24 | 16.1 | 2.6 | 81.4 | 0.0 | 0.0 |
| *Marrubium supinum* | 88.9 | 584 | 0.14 | 0.31 | 75.9 | 16.8 | 6.6 | 0.0 | 0.8 |
| *Phlomis lychnitis* | 45.0 | 137 | 0.00 | 0.06 | 96.9 | 3.1 | 0.0 | 0.0 | 0.0 |
| *Vicia villosa* | 52.6 | 2225 | 0.00 | 0.41 | 46.0 | 43.9 | 9.8 | 0.0 | 0.3 |

### *Estimated species richness*

Although the sampling effort was equivalent for all plant species (10 nectar drops per species), yeast abundance and frequency of occurrence differed widely among species (Herrera *et al.*, 2009), which explains the variation in the number of isolates obtained per plant species (Table 1) and justifies the application of rarefaction-based methods to obtain reliable estimates of total species richness.

When the whole sample of nectar drops from all plants species were combined into a single analysis, the species accumulation curve was close to reaching a plateau for the $N$ = 128 nectar samples examined (Fig. 2a). This finding reveals that, although additional rare species are expected to arise by further increasing the total sampling effort on the set of 24 plant species surveyed, results of this survey can be considered as providing a reliable basis for estimating overall yeast species richness in the floral nectar of this set of host species. Species richness estimates obtained from all nectar samples combined were 25.7 (ICE estimator) and 21 species (Chao2 estimator) (Fig. 2), which denotes that our sampling recovered around 50% of the total number of species occurring in the nectar of sampled plant species in the study area.

Rarefaction analyses were also conducted using plant species rather than individual nectar samples as the units for the analyses. Using this approach, the yeast species accumulation curve is not linear and does not reach a distinct plateau for the $N$ = 24 plant species sampled (Fig. 2c). The two nonparametric richness estimators, ICE and Chao2, gradually drifted apart from the Mao Tau function (observed diversity) with increasing number of plant species (Fig. 2d). The steadily increasing curves shown by these two estimators denote that our sampling of the plant community was able to detect only one third of the total estimated yeast species richness occurring in floral nectar in the regional plant community as a whole.

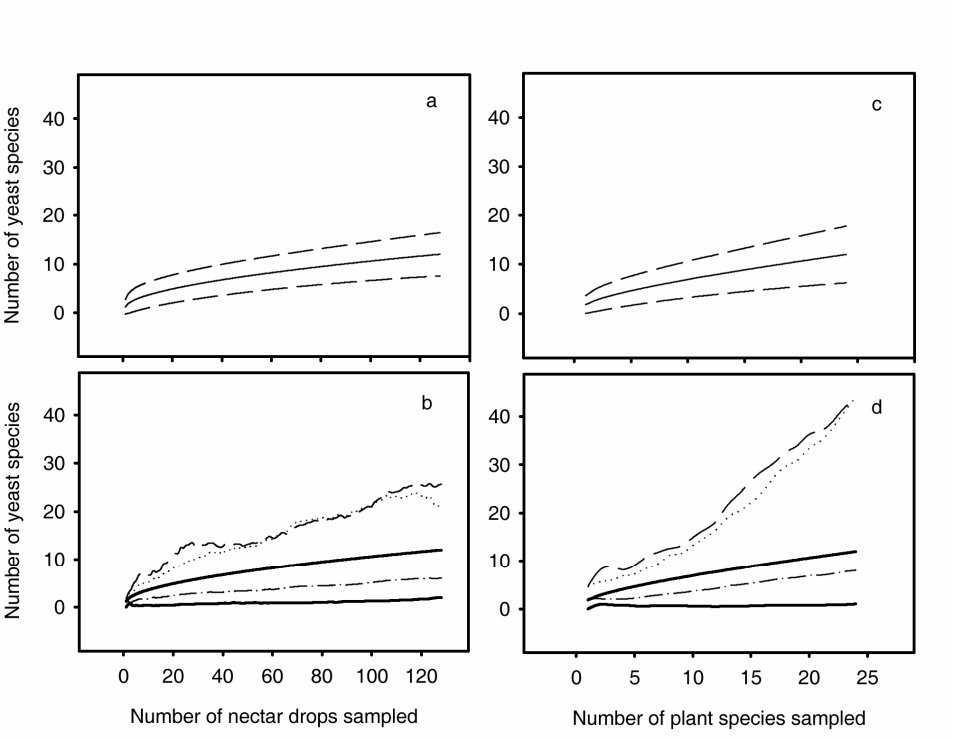


**Figure 2**. Species richness vs. species density. A) Individual-based rarefaction curve (black solid line, Mao Tau function) and 95% confidence intervals (long dash lines) for Cazorla 2008 spring nectar drops dataset. B) Performance of non-parametric estimators of species richness for Cazorla 2008 spring nectar drops dataset: Singletons (dash-dotted line), Doubletons (medium dash), ICE (long dash) and Chao2 (dotted line), in comparison with rarefaction curve (Mao Tau function, solid line). C) Individual-based rarefaction curve (black solid line, Mao Tau function) and 95% confidence intervals (long dash lines) for Cazorla 2008 spring dataset considering plants species surveyed as sampling units. D) Performance of non-parametric estimators of species richness for Cazorla 2008 dataset by plant species surveyed: Singletons (dash-dotted line), Doubletons (medium dash), ICE (long dash), and Chao2 (dotted line), in comparison with rarefaction curve (Mao Tau function, solid line). Yeast species appearance was based on incidence data.

### *Yeast community composition and osmotolerance*

Variation among yeast species in their frequency of occurrence in nectar drops or plant species (Fig. 1) was related to interspecific differences in physiological traits, with osmophilic species occurring most frequently. The magnitude of the effect differed slightly depending on whether species frequencies were computed on the basis of their occurrence in nectar drops or plant species.

When frequencies of occurrence were computed in respect to nectar drops, the set of osmophilic yeast species occurred, on average, in 27.7% of samples, while non-osmophilic yeasts occurred in 1.5% of samples, the difference being statistically significant ($P$ = 0.048; Wilcoxon rank-sum test). Frequency of occurrence across plant species was also significantly greater for the osmophilic yeasts, which on average occurred in 35.5% of plant species, as compared to non-osmophilic ones which occurred in only 4.8% of the plant species surveyed ($P$ = 0.024).

***Yeast diversity and plant pollinators***

Table 2 summarizes data for the subset of 12 plant species with information simultaneously available on yeast incidence and pollinator diversity and composition. Interspecific variation in yeast diversity and density was not significantly related to quantitative differences in pollinator composition ($r_s$ = 0.12, $P$ = 0.71 in both cases). Pollinator composition, as described by scores on the first principal component, correlated weakly with yeast frequency but the relationship did not reach statistical significance
($r_s$ = -0.448, $P$ = 0.14). No correlations were found between pollinator diversity, as computed with the Shannon Index, and yeast variables (frequency, density and diversity) ($r_s$ = 0.01-0.18).

## Discussion

As habitat for yeast growth, floral nectar is characterized by high cell densities (Herrera *et al.,* 2009; de Vega *et al.*, 2009) and low species richness (Brysch-Herzberg, 2004; Herrera *et al.*, 2010) in contrast to other microbial habitats such as aquatic environments that are characterized by high species richnesses López Arcilla *et al.* 2004) and low cell densities (Nagahama, 2006). This paper describes a particularly species-poor nectar yeast community, largely composed of only two species (*Metschnikowia reukaufii* and *M. gruessii*). The rest of species identified were rare in nectar samples, although some of them are common inhabitants in other environments like plant surfaces, soil, air and water (Barnett *et al.*, 1990, Sampaio *et*

*al.*, 2007). Species accumulation curves showed that further rare species would appear if more nectar samples from the 24 plant species surveyed were sampled, or if additional plant species were sampled, but the main conclusion would most likely remain unchanged that nectar yeast communities in our study region are characteristically species-poor.

A number of mechanisms may contribute to the low yeast species richness found in nectar communities (see also Brysch-Herzberg, 2004). Such factors may include the fact that we have used culture-dependent methods to obtain yeast isolates, irrespective of how the unknown pool of yeast species growing in nectars tolerate the growth medium's conditions. Nevertheless, culture-dependent methods are widely used for the characterization of yeast communities for clinical or ecological purposes, and recent studies on yeast diversity in natural conditions have revealed a better performance of culture-dependent methods over purely molecular techniques like temperature gradient gel electrophoresis or RFLP (Herzberg *et al.*, 2002; Gadanho and Sampaio, 2004). The existence of undescribed species could be another source of bias in our GenBank-querying species identification method, but results of DOTUR-based analyses tend to rule out this possibility as a major biasing factor. A total of 18 OTUS were identified at the 3% dissimilarity cut-off used, which is not dramatically different from the 12 species identified using BLAST queries. The low yeast species richness found in this study could also been explained by the fact that we focused exclusively on nectar samples rather than on flowers as a whole. Other surveys of flower-dwelling yeasts yielding longer species lists do not provide detailed information on which floral parts were sampled (e.g., Lachance *et al.,* 1999; Lachance *et al.*, 2001). In fact, a higher species richness is expected to occur at the level of whole flowers, since flowers harbour contrasting microhabitats for microbial growth such as pollen, perianth surfaces and nectaries. Soil or plant surfaces could represent macro-scales of sampling in relation to microbial body size (Fierer *et al.*, 2007), which might partly account for the high species richnesses revealed by surveys of these microbial habitats.

The predominance of ascomycetous over basidiomycetous yeasts found in this study probably reflects that, as a microhabitat, floral nectar favors fermentative, osmotolerant, copiotrophic species. This interpretation is supported by our analyses on the relationship between species occurrence and ability to grow in 50% glucose, and also agrees with some earlier predictions (Lachance, 2006). When interpreting the results of these analyses, however, it must be kept in mind that individual yeast species might be internally heterogeneous in respect to their responses to osmotic stress, and that focusing at the multi-specific level as done here misses such source of variation. Additional work on different yeast strains from the different species involved would be needed to explore the potential influence of that effect on our conclusions.

Two groups of yeast species could be distinguished, namely those few present in nearly all nectar samples and the majority that occurred in only a few plant species. The first group includes two specialist species in the *Metschnikowia* clade that have been frequently isolated from flowers, nectars and pollinator (Lachance *et al.,* 2001). The second group included generalist species that are found frequently in other microenvironments like leaf surfaces, soil, freshwater and air. These include species found in decomposing organic matter, phylloplane and soils (*Aureobasidium*) along with ubiquitous species of *Cryptococcus* and *Rhodotorula.* Additional work is needed to dissect the different ecological origins of yeast species found in floral nectar, discerning which species originate from pollinators' glossae, pollinators body surfaces, corolla inner surface or pollen.

Although rarefaction curves do not provide an estimation of asymptotic species richness (Tipper, 1979; Mao *et al.*, 2005), they are often used to evaluate sampling adequacy by assessing whether the cumulative number of species reaches a plateau, and to compare observed species richness with figures obtained with nonparametric estimators (Colwell and Coddington, 1994; Ugland *et al.*, 2003; Colwell *et al.*, 2004). Nonparametric richness estimators, infrequently used in microbial diversity estimates (Hughes *et al.*, 2001, and references therein), may be used for inferring true species richness (Walther *et al.*, 1995). As shown in this

study, observed species richness can be substantially lower than the values predicted by the ICE and Chao 2 estimators, thus corroborating that observed species accumulation curves will frequently underestimate actual species richness (Colwell and Coddington, 1994). Nonparametric estimators incorporate information on the distribution of rare species in the dataset (i.e., those represented once, twice or only a few times), so the fact that the majority of species recovered from our samples were rare account for the marked dissimilarity found here between observed and predicted species richness.

Rarefaction curves did not reach a plateau, thus revealing that additional sampling effort would be needed to assess the true diversity of nectar yeasts in the study area. The total species diversity in a given area can be broken down into within-habitat or alpha-diversity, and between-habitat or beta-diversity (Magurran, 2004). Since alpha-diversity refers to a group of organisms interacting and competing for the same resources or sharing the same environment, results obtained from our drop-based analyses would roughly correspond to this diversity component. In contrast, plant species-based approach would fall conceptually closer to beta-diversity, allowing comparisons between different ecosystems or along environmental gradients (Magurran, 2004). Because of practical limitations, total sampling effort was restricted to 150 nectar drops spread among plant species. If nectar yeast communities were characterized by high beta-diversity, which would imply a high species turnover between plant species, then maximizing the spread of nectar drops across plant species would lead to an optimal sampling design. The use of individual plant species as units of analysis achieved lower accuracy at assessing true diversity at the plant community level than the use of pooled nectar drops, which is not surprising given that the number of samples involved in the nectar drops approach was considerably larger than in the plant species approach. Considering also that each plant species comprises a large array of elemental nectar "habitats", using species as sampling units may provide an excessively coarse scale for estimating species richness of nectar yeast communities at the habitat level unless a much larger number of nectar samples is collected from each plant species.

Although a number of microbiological surveys of nectar yeasts have been carried out previously (e.g., Jimbo, 1926; Sandhu and Waraich, 1985; Eisikowitch *et al.,* 1990a; Herzberg *et al.*, 2002; Brysch-Herzberg 2004), direct comparisons between our survey and previous work are problematical, since species identifications in the early literature on nectar yeasts was mostly based on traditional microbiological tests (e.g. Caprioti, 1953; Sandhu and Waraich, 1985; Vörös-Felkai, 1957). The list of genera recorded in our study is closely similar to those reported for *Asclepias syriaca* nectar in North America and a broad survey of nectar yeasts in central European plants (Brysch-Herzberg, 2004; Eisikowitch *et al.,* 1990a) Among recent work using molecular identification methods, the study of Herzberg *et al.* (2002) in Germany remains the most thorough investigation to date on nectar yeasts where the majority of yeast identifications were based on DNA sequences. Results reported in this study and those of Brysch-Herzberg for Germany are similar in that *Metschnikowia reukaufii* was the commonest species at both sites (65% and 47 % of nectar samples in southern Spain and Germany, respectively) followed by *M. gruessii* (23% and 17%). Species of *Cryptococcus* were isolated more frequently in Germany (18% of samples, compared to only 2% in southern Spain). The published data for Germany are amenable to rarefaction analysis, and a comparison of sample-based rarefaction curves for the two regions is shown in Fig. 3. The two species accumulation curves bear strong resemblance and, although similarly low at both regions, observed species richness was slightly higher in Germany than in southern Spain.

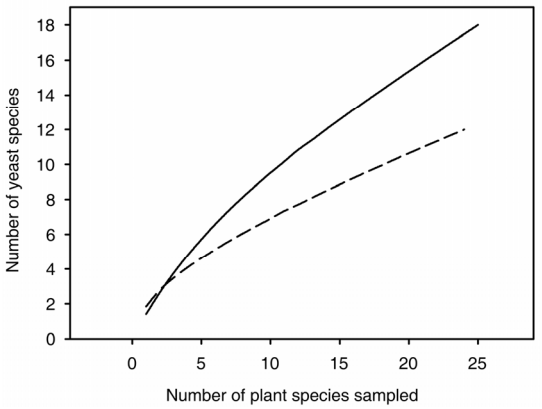


**Figure 3**. Comparison of sample-based rarefaction curves from this study (dashed line) and a comparable investigation on the nectar-dwelling yeasts from a set of 25 plant species surveyed in Germany by Herzberg (2002) (solid line).

Although further studies are obviously needed, the provisional conclusion can be drawn from these two studies (see also Caprioti, 1953; Voros-Felkai, 1957) that European nectar yeast communities are characterized by generally low species richness and a marked numerical dominance by very few species, represented here by *M. reukaufii* and *M. gruessii*. The ecological mechanisms accounting for these patterns remain to be elucidated, but factors related to variable, species-specific colonization opportunities, interspecific antagonistic relationships, and growth limitations imposed by osmotic stress, low nitrogen content and presence of secondary compounds in the nectar (Herrera *et al.*, 2010) could all play some role in determining the composition and diversity of nectar yeast communities.

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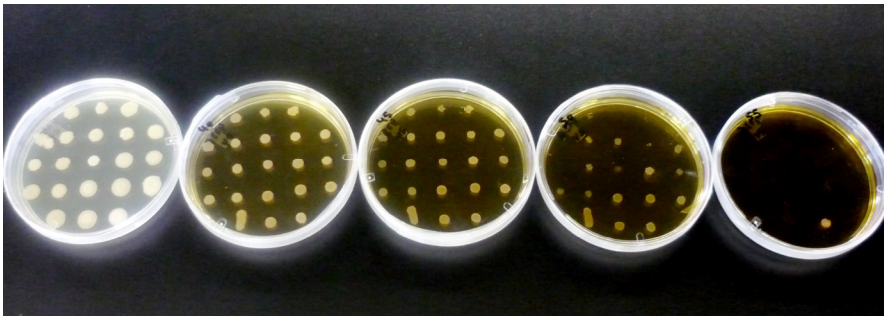
# Appendix

**Distribution among families of the 24 angiosperm species whose floral nectar was examined microscopically in this study for the identification of yeasts. Familial classification follows (APG, 2003).**

| Plant Family | Species surveyed |
|---|---|
| **Fabaceae** | *Anthyllis vulneraria, Erinacea anthyllis, Tetragonolobus maritimus, Vicia onobrychioides, Vicia villosa* |
| **Lamiaceae** | *Marrubium supinum, Phlomis lychnitis, Prunella grandiflora, Teucrium pseudochamaepitys.* |
| **Plantaginaceae** | *Antirrhinum australe, Digitalis obscura, Linaria aeruginea, Linaria lilacina* |
| **Iridaceae** | *Gladiolus illyricus, Iris foetidissima, Iris pseudacorus, Iris xiphium* |
| **Caprifoliaceae** | *Lonicera etrusca, Lonicera implexa* |
| **Ranunculaceae** | *Aquilegia vulgaris, Aquilegia cazorlensis* |
| **Brassicaceae** | *Erysimum myriophyllum* |

# The role of immigration and physiological traits in nectar yeast community assembly.



# CAPÍTULO 4

*El papel de la inmigración y los rasgos fisiológicos en la formación de las comunidades de levaduras del néctar.*

# CAPÍTULO 4

## The role of immigration and physiological traits in nectar yeast community assembly.

*El papel de la inmigración y los rasgos fisiológicos en la formación de las comunidades de levaduras del néctar.*


## Abstract

Recent studies have shown that dense yeast populations often occurring in floral nectar are numerically dominated by a few species in the genus *Metschnikowia*, while generalist yeasts commonly occurring on leaf surfaces, soil, freshwater, and air were rarely isolated from nectar samples. A study was designed to understand the main factors responsible for the assembly of nectar yeast communities, by combining field experiments with laboratory tests of the physiological abilities of all yeast species forming the pool of potential colonizers for two Spanish flowering plants (*Digitalis obscura*, *Atropa baetica*). Yeast frequency and species richness were assessed in external sources (bee glossae, air, plant phylloplane) as well as in pollinator rewards (pollen, nectar). Yeasts were most frequent in external sources (air, flower visiting insects), less so in the proximate floral environment (phylloplane), and least in pollen and nectar. Nectar communities were considerably impoverished versions of those in insect glossae and phylloplane. Nectar, pollen, and insect yeast assemblages differed in physiological characteristics from those in other substrates. Nectarivorous *Metschnikowia* were not more resistant than other species to plant secondary compounds and high sugar concentrations typical of nectar, but their higher growth rates may be decisive for their dominance in ephemeral nectar communities.

## Resumen

Según estudios recientes, el néctar floral a menudo contiene densas poblaciones de levaduras. Estas comunidades están compuestas mayoritariamente por unas pocas especies del género *Metschnikowia*, junto con otras levaduras generalistas comúnmente aisladas de las hojas, suelo, agua dulce y aire, si bien estas últimas son aisladas en una frecuencia mucho menor dentro del néctar. Este estudio trata de entender los principales factores que afectan al ensamblaje de las comunidades de levaduras del néctar floral, y para ello combina experimentos de campo con pruebas de laboratorio sobre las propiedades fisiológicas de todas las especies de levaduras que componen el conjunto de potenciales colonizadoras del néctar. Así, para dos especies de plantas del Sur de España, *Digitalis obscura* y *Atropa baetica*, se valoró tanto la frecuencia de muestras con levaduras como la riqueza de especies presentes en las posibles fuentes externas de levaduras hacia el néctar (piezas bucales de insectos visitantes, aire, filosfera), así como en las recompensas florales (polen, néctar). Las levaduras fueron más frecuentes en las fuentes externas (aire, insectos) que en el filoplano, el polen y el néctar. Las comunidades de levaduras del néctar fueron versiones empobrecidas de las comunidades asociadas a las glosas de los insectos polinizadores y el filoplano. Las levaduras provenientes de néctar, polen y glosas de insectos se caracterizaron por perfiles fisiológicos diferenciados respecto al resto de substratos analizados. Las principales especies del néctar (*Metschnikowia*) no fueron más resistentes que otras especies a los compuestos secundarios de las plantas, ni a las altas concentraciones de azúcar típicas del néctar, pero su alta tasa de crecimiento sí podría explicar su clara dominancia en las comunidades de levaduras del néctar.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## Introduction

Microbial community dynamics on plant parts have been studied for over a century (e.g. Ruinen, 1963; Last and Price 1969; Phaff and Starmer 1987, Thompson *et al.*, 1993; Lindow and Brandl, 2003, Sampaio *et al.*, 2007), but the ecological processes that generate the observed patterns still remains to be elucidated (Kinkel, 1997). Individual plants provide a multitude of microhabitats with different topographical features, nutrients, water availability, and a range of microclimatic conditions which in turn help establish correspondingly diverse microbial communities (Andrews and Harris, 2000). Some yeast microhabitats within plants have been described as specific. These include decaying tissues, flowers, nectar, or fruits (Phaff and Starmer, 1987; Lachance *et al.*, 1989, Starmer *et al.*, 1990; Rosa *et al.*, 1992; Spencer *et al.*, 1992, Rosa *et al.*, 1994).

In some studies carried out recently in SE Spain, yeast communities of nectar were shown to possess high population densities and a clear predominance of members of the genus *Metschnikowia* (Herrera *et al.*, 2009; Herrera *et al.*, 2010; Pozo *et al.*, 2011). The few additional yeasts found in nectar consisted of generalist species that are known to occur in other microenvironments such as leaf surfaces, soil, freshwater, and air (Herzberg *et al.*, 2002; Brysch-Herzberg, 2004).

Microbial population dynamics in floral nectar, as in any other habitat, may be considered a complex function of four processes: immigration, emigration, growth, and death (Fonseca and Inacio, 2006). It is therefore reasonable to ask whether floral nectar is depleted at the same rate as the overall incoming yeast community or whether it represents a set of identical individual microhabitats that constrain colonization to a small group of highly specialized yeast species. In comparison to other plant parts, spring or summer flowers have a very short lifespan. Inside the flowers, nectar is a highly fluctuating habitat that experiences evaporation at high temperatures, dilution by rain, and depletion from pollinator visits. Nectar colonization by yeasts is constrained by their close association with the flower visiting insects, which both introduce the yeasts through their mouthparts and consume them along with the nectar (Gilbert, 1980; Sandhu and

Waraich, 1985; Brysch-Herzberg, 2004; Canto *et al.*, 2008). The complex interaction associated with colonization history is compounded further with yeast survival and growth processes. On the one hand, the high sugar content common to floral nectars (Nicolson and Thornburg, 2007) may serve as a source of nutrients that can support microbial development and at the same time may render the habitat selective in favour of highly osmotolerant yeasts. Furthermore, nectarivorous diets are also characterized by a low nitrogen content (Nicolson, 2007). On the other hand, nectars of many plant species contain plant secondary compounds (Adler, 2000, Manson *et al.*, 2007, Nicolson *et al.*, 2007, Gonzalez-Teuber and Heil, 2009) that are thought to act as a deterrent for nectar robbers (*e.g.*, inefficient pollinator insects, nectar-consuming microbial communities), thus favouring "legitimate" visitors. In this context, yeasts deplete nectar sugars, devaluate the floral reward (Herrera *et al.*, 2008), and consequently might be affected by the presence of these substances. In plant species that produce defensive compounds, as is the case with those used in this study, these compounds may limit the number of yeast species that finally constitute nectar communities. The failure of microorganisms to survive in floral nectar may then be the result of nectar physical conditions themselves, as it has been noted above, or to competition processes. Given that floral nectar is both an overpopulated (Herrera *et al.*, 2009) and a resource-limited habitat, the competitive advantage of *Metschnikowia* species may also be the result of a superior rate of reproduction and survival, which in turn leads to a reduction in competitors.

The main objective of this study is to determine as comprehensively as possible the origin and composition of the yeast species pool potentially arriving into floral nectar in two different plant species at the plant population scale. To this end, several microhabitats are evaluated as potential sources of yeast to floral nectar, and yeast frequencies are measured at different levels following a hierarchical sampling scheme in each individual plant. The levels range from external sources such as flower visiting bee glossae, air, bracts, and corolla surfaces, to floral rewards such as nectar and pollen. Measures of yeast species transference

to floral nectar are indicative of the degree of microsite specialization, which raises the question of the mechanisms underlying nectar yeast community assemblage. Accordingly, we then examine if interspecific differences in carbon and nitrogen sources utilization patterns, resistance to inhibitors and plant defensive compounds, osmotolerance, and growth rate contribute also to explaining features of the nectar yeast community.

## Materials and Methods

### *Field sampling*

A total of 234 yeast isolates were obtained from the two study species, willow-leaved foxglove (*Digitalis obscura*, Plantaginaceae; *Digitalis* hereafter) and *Atropa baetica* (Solanaceae; *Atropa* hereafter), in 2009, in the Sierra de Cazorla region, a well-preserved natural area in Jaén province, Southeastern Spain. Each plant species contributed one population and the two were sampled sequentially according to their blooming period, which ranges from early June to late July. Samples were collected from each locality within 4 consecutive days. The *Digitalis* sampling site contributed 162 yeast isolates and the other 72 were recovered from *Atropa*. The two localities were located 7.9 km apart.

Yeast isolates were obtained in a spatially nested scheme that ranged from external sources such as bees' glossae and air samples to plant-related samples obtained from 20 randomly selected plant individuals in each sampling site. We sampled two flowers from each individual, from which we systematically collected one subsample each from bracts, corolla outer and inner surfaces, pollen, and nectar samples. In order to avoid artifacts caused by different yeasts colonization times, we systematically collected mature flowers that were 3 days old. *Digitalis* plants produce protandrous flowers that last for up to 5 days. In contrast, flowers of *Atropa* are markedly protogynous and last for up to 3-4 days. Nectar production at both species tend to be higher at the intermediate phase of floral development,

which coincided with the female phase in *Digitalis*, and the male phase in *Atropa* flowers (MIP, *pers. obs.*).

We performed three different treatments. The first consisted of naturally exposed to insect visitation flowers of ten individuals in each sampling site and the second of flowers of ten other plants excluded from pollinator visitation by bagging at the bud stage (*N*= 20 flowers in each treatment; "exposed" and "insect visitors excluded", hereafter). After completing *Digitalis* experiments, we realized that nectar from insect visitors-excluded plants still harboured yeast in a significant fraction, and so we covered two additional buds in each of the ten previosly bagged plants with cellophane envelopes ("airflow prevented" samples, (*N*= 20 flowers).

Eighteen and fifteen bees were hand-netted while they foraged on *Digitalis* and *Atropa* sampling sites, respectively. Three *Bombus* species were collected from *Digitalis* plants (*B. pascuorum*, *B. pratorum,* and *B. terrestris*), comprising both workers and males; and *B. terrestris* (males), *B. pascuorum* (workers), plus the solitary bees *Anthidium florentinum* and *Anthophora* (*Amegilla) quadrifasciata* were recorded in *Atropa* flowers. Based on pollination censuses carried out simultaneously for both sampling sites (see Herrera *et al.*, 2001 for censuses methodology) both bee pools were a representative sample of the whole set of visitors (Table S1). Immediately upon capture, bees were placed individually in sterile containers and anaesthetized by placing them for two minutes inside a freezer at -20ºC. The glossa of each individual bee was carefully extended, using sterile forceps, beyond the tip of the maxillary galeae and carefully rubbed against the surface of an agar plate. Airborne yeast samples were obtained by placing an open agar plate under each selected plant for five minutes.

Entire flowers and their most proximate bract were collected in the field and kept refrigerated in a sterile container until brought to the lab. The bracts and corollas were gently swabbed for inoculation on agar medium. Inner and outer surfaces of the corollas were also sampled by imprinting them onto agar medium. Pollen was sampled by immersing a mature anther in 1 ml sterile water for 48h and streak-inoculating 1 μl of this inoculum onto an agar plate. Nectar samples were

taken with 1 µl calibrated microcapillaries and transferred onto agar plates for streak inoculation.

***Yeast isolation, identification and physiological characterization***

All samples were cultured onto yeast malt agar plates (1.0% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract, and 2.0% agar) with 0.01% chloramphenicol and incubated at 25°C.

Isolates were purified and characterized following the standard methods of Yarrow (1998). Growth responses were evaluated by replica plating onto 115 test media developed by Lachance (1987) or specifically conceived for this study. This included growth on 44 carbon sources, 20 inhibitors, five nitrogen sources, four fermentation tests, three tests of hydrolysis, three halotolerance tests, three tests of morphological characteristics, two carbon and nitrogen sources tests, two growth factors, and individual tests for osmotolerance, acid production, colour reaction, and starch production, and 25 growth temperatures. The D1/D2 domains of the large subunit ribosomal RNA gene was two-way sequenced for all the isolates as described in Lachance *et al.* (1999), using the primers pair NL1/NL4. Gblocks (Castresana, 2000) was used to trim the resulting alignment and the DNA sequences were compared with those in the GenBank database (last accessed 9 June 2010) by querying with the BLAST tool. Using the same procedure as Pozo *et al.* (2011), we also evaluated Operational Taxonomic Units (OTUs) defined on the basis of DNA sequence similarity with the program DOTUR, using the 3% similarity threshold (Distance-Based OTU and Richness, Schloss and Handelsman, 2005).

***Plant secondary compounds assays***

Agar diffusion was used to evaluate the possible effect of plant defensive compounds present in nectar on yeast growth. We tested three of the major compounds in each plant, namely the tropane alkaloids atropine, tropine, and scopolamine from *Atropa* (Adler, 2000, Zárate *et al.*, 2007) and the digitoxose-type cardenolides digitoxin, digitoxigenin, and gitoxigenin from *Digitalis* (Gavidia and

Pérez, 1997; CM Herrera, *unpublished data*). The potential effect of these compounds was assessed on 12 yeast strains, each from a different species: *C. bombi, C. floricola, C. laurentii, C. victoriae, D. maramus, L. thermotolerans, M. reukaufii, M. gruessii, M. kunwiensis, R. colostri, S. bombicola,* and *S. roseus*. Plates of Yeast Nitrogen Base (YNB, Difco) plus 1% of glucose and 1.5% agar were inoculated with dilute yeast suspensions of each yeast strain, and 5 μl of dimethyl sulfoxide (DMSO) solutions containing 1 mg of each compound were added as individual drops. Lack of toxicity of DMSO was corroborated for yeast strains by exposing yeasts to 5 μl of this solvent. The plates were incubated at 24ºC and examined periodically for evidence of inhibition zones induced by secondary compounds.

***Osmotolerance and optimal growth temperature assays***

The growth responses to increasing proportions of glucose in agar media was tested for 144 strains belonging to the 29 yeast species recovered from the different microhabitats studied. The media contained 1% yeast extract, 1.5% agar, and 40, 45, 50 or 55% (w/w) glucose, and the plates were incubated at 24ºC. Growth of the same strains was evaluated on YM agar at four, six, eight, 10, and 12ºC and from 26 to 42ºC with increments of one degree. Yeast growth was recorded on an ordinal scale after three, seven and 18 days. For taxa represented by more than one specimen, the most common response is reported.

***Data analyses***

Sample-based occurrence data were used to obtain observed species richness (Mao Tau function, Mao *et al.*, 2005) and to calculate species richness estimators (ICE and Chao2) with EstimateS Version 8.2 (Colwell, 2005). This program was used also to conduct analyses of shared species between microhabitats using sample-based abundance data. The Chao shared estimator was used to compare species co-ocurrence between microhabitats. For these analyses we considered bee glossae and air samples together as "external sources" samples, and bracts and the two corolla surfaces as "phylloplane" samples. Pollen and nectar isolates were combined as "pollinator rewards" samples. For the purpose of

analyzing the physiological features of the yeast isolates, an 82-test subset from the replica-plating series was selected by choosing those tests for which more than 5% of the strains differed in growth response. This subset comprised 39 carbon sources, 16 temperatures, 12 inhibitors, five nitrogen sources, three halotolerance and one osmotolerance tests, three tests of lytic activity, one test of carbon and nitrogen source, and individual tests for vitamin independence and glucose fermentation. The growth responses for these tests for 50 yeast strains were analysed using Principal Component Analysis (PCA) with cross-validation on the covariance matrix (Statistica 7.0, StatSoft). Interpretation of the data was made by inspection of the scores and loadings plots for the first two components, which accounted for 41% of the total variance.

Twenty-nine yeast species were clustered according to growth responses to increasing sugar concentrations by calculating the Euclidean distance matrix as implemented in PCORD v 4.0 Clustering Analysis. An UPGMA tree was computed using PHYLIP Neighbour on this distance matrix.

In view of the temperature range to which yeasts were naturally exposed (CM Herrera, *unpublished data*) at both sampling sites during the sampling period, yeast growth slopes were represented from eight (minimum field temperature during experiments) to 30 degrees (maximum). Although yeast growth was followed over 18 days, considering the ephemeral nature of flowers as yeast habitats, and more specifically, that flowers of *Digitalis* and *Atropa* last up 4-5 days (MI Pozo, *unpublished data*) only the data on growth rate within three days of incubation at each temperature were included in the analyses.

## Results

### *1. Occurrence of yeast species in different microhabitat types*

The frequency of samples containing yeasts was the highest in external sources such as air samples and bees (above 70%), less so in samples belonging to the proximate environment of the flower, and the lowest in nectar and pollen

samples, even though the two plant species studied differed in yeast frequency at each microsite (Fig. 1).

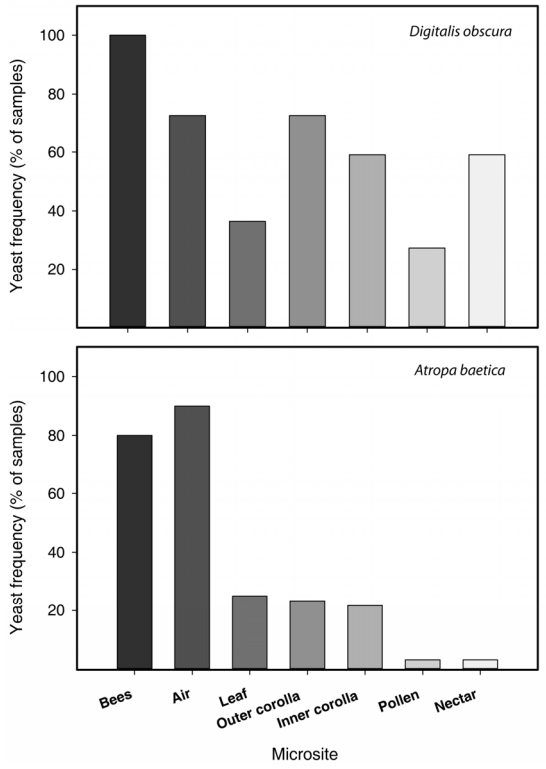


**Figure 1.** Frequency of samples containing yeasts in each microhabitat for the two sampling sites studied.

In plants with insect visitors excluded, phylloplane samples continued carrying roughly the same proportion of yeasts, but pollen samples harboured yeasts at a 50% lower frequency (Fig. 2). The two plant species showed different patterns with respect to nectar yeast frequency in excluded and exposed treatments. Exposed *Digitalis* nectar contained yeasts in 60 % of samples, this figure falling to 30% when insect visits were prevented. Nevertheless, in *Atropa* nectar yeasts were only recovered in bagged flowers. When both airborne microbial contamination and pollinator visits were prevented at the same time, no yeasts were recovered

from pollen and nectar samples, and corolla surfaces carried yeast in <15% of samples.

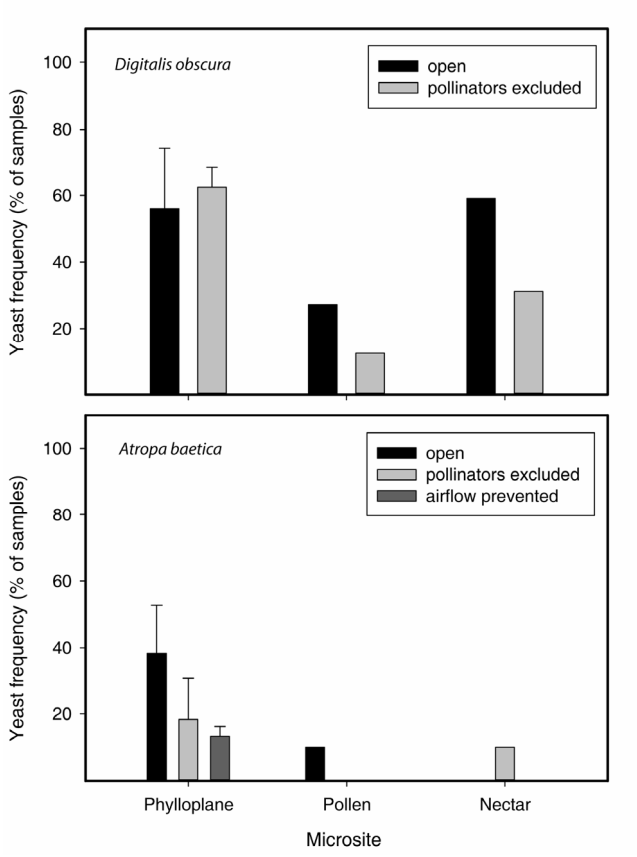


**Figure 2.** Frequency of samples containing yeasts in the phylloplane (both corolla surfaces and bract mean±SD), pollen, and nectar samples from the two plant species in sequential bagging experiments: exclusion of pollinator-vectored yeasts (upper panel) and airborne plus pollinator-vectored yeasts (bottom panel).

### *2. Spatial distribution of yeast species within plants*

The 234 yeast isolates obtained at the two study sites belonged to 17 genera and 36 species (Table 1). Both plant species were characterized by similar amounts of yeast species richness, with 22 (*Digitalis*) and 23 (*Atropa*) species, respectively, in total. The DOTUR-based analysis of DNA sequences yielded fairly similar results in *Atropa* sampling site, with a total of 26 OTUs. In *Digitalis,* the method recognized

up to 38 distinct OTUs, which suggests that some undescribed species may occur in the samples.

**Table 1.** Species of filamentous fungi and yeasts recovered from air, bee glossae, phylloplane, nectar, and pollen samples, as a function of the plant species surveyed. Shaded boxes indicate the presence and the number of isolates of a species in a given sample type.

| | *Digitalis obscura* | | | | | | | *Atropa baetica* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Fungal spp.** | Bees | air | bract | outer corolla | inner corolla | pollen | nectar | bees | air | bract | outer corolla | inner corolla | pollen | nectar |
| *Aureobasidium pullulans* | 1 | 3 | 14 | 21 | 11 | 2 | 7 | 1 | 5 | 5 | 1 | 1 | | |
| *Candida bombi* | | | | 3 | | | | | | | | 1 | | |
| *Candida floricola* | | | | | | | | 5 | | | | | | |
| *Candida friedrichii* | | | | | 1 | | | | | | | | 1 | |
| *Coniochaeta leucoplaca* | | | | | | | | | | | | | | 1 |
| *Cryptococcus adeliensis* | | 1 | | | | | | | | | | | | |
| *Cryptococcus aerius* | | 5 | | 1 | | 1 | | | | | | | | |
| *Cryptococcus festucosus* | | | | 1 | | | | | 1 | | 1 | 1 | | |
| *Cryptococcus laurentii* | 1 | | | | | | | | | | | | | |
| *Cryptococcus magnus* | | | | | | | | | | | 1 | 1 | | |
| *Cryptococcus oeirensis* | | | 1 | | | | | | | 1 | 2 | 1 | | |
| *Cryptococcus saitoi* | | | | 1 | | | | | 1 | | | | | |
| *Cryptococcus stepposus* | | | | | | | | | 2 | | | 2 | | |
| *Cryptococcus terreus* | | 1 | | | | | | | | | | | | |
| *Cryptococcus victoriae* | | | | | 1 | | | | 3 | 2 | 2 | | | |
| *Debaryomyces hansenii* | | | | | 1 | | | | | | | | | |
| *Debaryomyces maramus* | 1 | | | 1 | 1 | | | | | | 1 | | | |
| *Debaryomyces nepalensis* | | | | | | | | 2 | | | | | | |
| *Dothichiza pythiophila* | | | | | | | | | | 1 | | | | |
| *Dothiora elliptica* | | | | | | | | | | | 1 | | | |
| *Lachancea thermotolerans* | | | | | | | | 1 | | | | | | |
| *Metschnikowia gruessii* | 21 | | 2 | 2 | 4 | 6 | 19 | | | | | | | |
| *Metschnikowia kunwiensis* | 1 | | | 2 | 4 | | | | | | | | | |
| *Metschnikowia reukaufii* | 11 | | | | | | | 12 | | | | 1 | 1 | |
| *Ogataea zsoltii* | | | | | 1 | | | | | | | | | |
| *Rhizosphaera pini* | | | | 1 | | | | | | | | | | |
| *Rhodotorula mucilaginosa* | | | | | | | | | | | | | | 1 |
| *Rhodotorula sp.* | | 1 | | | | | | | | | | | | |
| *Starmerella bombicola* | 1 | | | | 2 | | | | | | | | | |
| *Sydowia polispora* | | | | | | | | | | 1 | 1 | 1 | | |
| *Trichosporon montevidensis* | | | | | | | | | | | | 1 | | |
| *Trichosporon moliniforme* | | | | | | | | | | | 1 | | | |
| *Ustilago maydis* | | | 1 | 1 | | | | | | | | | | |
| *Ustilago sp.* | | | | | | | | | | | 2 | 1 | | |
| *Zygosaccharomyces mellis* | 1 | | | | | | | | | | | | | |
| *Zygosaccharomyces rouxi* | | | | | | | | | | | 1 | | | |
| **Number of distinct species** | 8 | 5 | 4 | 10 | 9 | 3 | 2 | 5 | 5 | 5 | 11 | 10 | 2 | 2 |
| **Number of yeast isolates** | 38 | 11 | 18 | 34 | 26 | 9 | 26 | 21 | 12 | 10 | 14 | 11 | 2 | 2 |

The yeast assemblages associated with the two plants were similar in showing a marked numerical dominance of a few species, namely the ubiquitous

phylloplane species *Aureobasidium pullulans* plus *Metschnikowia gruessii* that was recovered from all microhabitats except the air (Table 1). *Metschnikowia reukaufii* was frequently recovered from bees, and *Cryptococcus* species dominated the air and the phylloplane (Fig. 3).

Species richness did not follow the same pattern as did the yeast frequencies in samples. Bee glossae harbored eight and five yeast species in *Digitalis* and *Atropa,* respectively, and five yeast species were recovered from air samples at both sites. The highest yeast species richness was found in corolla samples and the lowest was in pollen and nectar, with two species in each substrate at both sites. Rarefaction analyses showed that the coverage of yeast species could be generally improved by additional sampling, except for *Atropa* local phylloplane and *Digitalis* nectar, whose curves appear to have reached a plateau with the existing sampling effort (Fig. 3).

***3. Comparative composition of yeast assemblages in plants, insects and air samples***

Yeast communities isolated from corolla surfaces at the two localities were remarkably similar, judging by the total number of the Chao-shared estimated species (7). In addition, the yeasts of bracts and bee glossae local sets would share up to two yeast species when comparing the two sampling sites.

When considering the two sampling sites separately, we also used the presence of shared species to study patterns at yeast species degree of interchange between microsites. The two plant species were characterized by different patterns of species similarity between microsites. In *Digitalis*, bee glossae and phylloplane samples shared up to eight yeast species. Some pollen yeast species would be shared (up to two) with phylloplane, bee glossae, and nectar samples. In *Atropa*, phylloplane and air samples were highly similar in species composition, with 31 shared species. In this sampling site, bee visitors would exchange a low proportion of species with phylloplane (1-2).

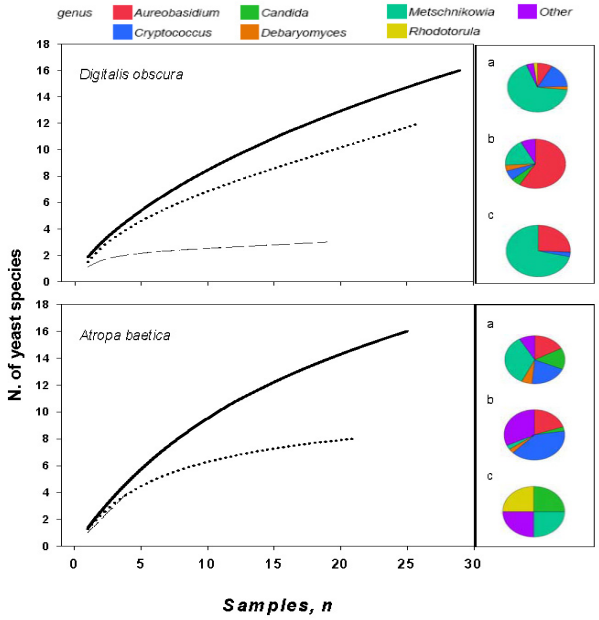


**Figure 3.** Species accumulation curves (rarefaction analysis) separately by microhabitats in *Digitalis* and *Atropa* localities: external sources (solid lines), phylloplane (dotted lines) and pollen and nectar communities (long dash lines). Pie charts display the taxonomic distribution of isolates among genera at each microhabitat compartment (external sources, a; phylloplane, b; pollen and nectar, c).

### *4. Microsites and physiological profiles*

The relationship between physiological profile and microsite of origin of yeast isolates was explored by Principal Component Analysis. The first two axes accounted for 41% of the total variation. The main factors underlying the first component (24% of variance, PC1, Fig. 4) were growth on L-arabinose, 2-ketogluconate, inositol, raffinose and gluconate ($r>0.7$). The second component (PC2, 17% of total variance) was correlated with nitrate, nitrite, and phenylalanine ($r>0.4$) as nitrogen sources and was moderately correlated with lipid hydrolysis and growth on D-glucoranate (see Supplementary material for further information). Yeast species did not show a well-defined clustering in a two-dimensional space defined by the first two axes of the PCA, but there is a microhabitat-aggregation

trend, in which those strains from air and phylloplane tend to disaggregate from those strains recovered from nectar, pollen and bees' glossae.

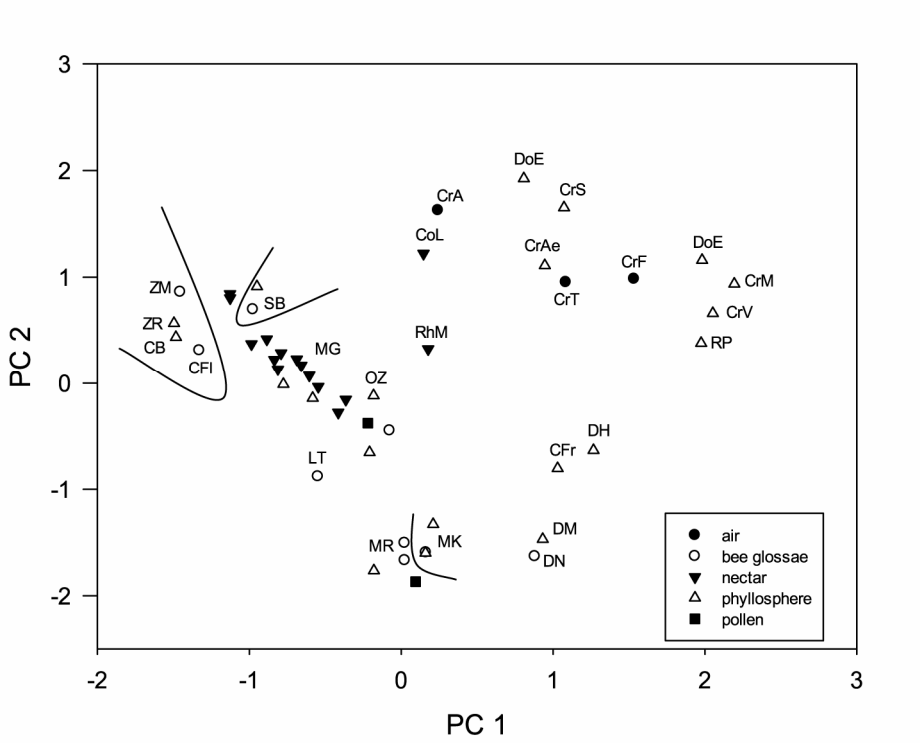


**Figure 4.** Plot representing the Principal Components Analysis of the physiological characteristics of 50 yeast strains and their natural microsite of origin. The first two principal components (PC1 and PC2) accounted for 41 % the total variation observed. The fungal species represented here are *C. bombi* (CB), *C. floricola* (CFl), *C. friedrichii* (CFr), *C. leucoplaca* (CL), *C. adeliensis* (CrA), *C. aerius* (CrAe), *C. festucosus* (CrF), *C. magnus* (CrM), *C. terreus* (CrT), *C. victoriae* (CrV), *D. hansenii* (DH), *D. maramus* (DM), *D. nepalensis* (DN), *D. pythiophila* (DoP), *D. elliptica* (DoE), *L. thermotolerans* (LT), *M. gruessii* (MG), *M. kunwiensis* (MK), *M. reukaufii* (MR), *R. mucilaginosa* (RhM), *O. zsoltii* (OZ), *S. bombicola* (SB), *Z. mellis* (ZM) and *Z. rouxii* (ZR). Curved lines are drawn on chart to separate yeast species when needed.

### *5. Osmotolerance and response to secondary compounds.*

We did not find any noticeable heterogeneity among yeast species in their resistance to six secondary compounds assayed in the subset examined. Cardenolides from *Digitalis* did not induce any inhibitory response irrespective of dose or strain tested. As to alkaloids, all the yeast species tested were equally

susceptible to concentrations of atropine and tropine above 150 ppm. Contrary to our initial hypothesis, *Metschnikowia* species did not exhibit a greater resistance to the toxic compounds assayed. In fact, only *M. kunwensis* and *M. reukaufii* strains were sensitive to Scopolamine 150 ppm.

**Table 2.** Comparative evaluation of osmotolerance of 13 yeast species[a] based on their growth response[b] on media containing 40 to 55% glucose.

| Fungal spp. | Association with high sugar environment (bee and/ or nectar) | Growth index with increasing glucose concentration (%w/w) | | | |
|---|---|---|---|---|---|
| | | 40% | 45% | 50% | 55% |
| *Debaryomyces nepalensis* | B | 5 | 5 | 5 | 5 |
| *Zygosaccharomyces mellis* | B | 5 | 5 | 5 | 4 |
| *Zygosaccharomyces rouxii* | | 5 | 5 | 5 | 4 |
| *Candida bombi* | | 5 | 5 | 3 | 3 |
| *Debaryomyces maramus* | B | 5 | 5 | 4 | 2 |
| *Metschnikowia kunwiensis* | B | 6 | 5 | 3 | 2 |
| *Candida floricola* | B | 5 | 5 | 5 | 0 |
| *Metschnikowia reukaufii* | B | 5 | 5 | 4 | 1 |
| *Starmerella bombicola* | B | 5 | 4 | 3 | 3 |
| *Lachancea thermotolerans* | B | 5 | 5 | 3 | 1 |
| *Metschnikowia gruessii* | B + N | 5 | 5 | 3 | 1 |
| *Candida friedrichii* | | 5 | 4 | 3 | 1 |
| *Debaryomyces hansenii* | | 5 | 4 | 3 | 1 |

[a] The present list is a subset of the 29 yeast species used in osmotolerance test. The rest ones, which are those not capable to grow in media containing 40% glucose, are listed in Fig. S1.
[b] Growth index: missing data, 0; absence of growth, 1; weak, 2-4; apparent growth, 5-6.

Osmotolerance tests revealed that 16 of the 29 yeast species examined failed to grow at 40% glucose. A great gradation in yeast growth response under increasing glucose concentrations may be discerned for the subset of osmotolerant yeast species (Table 2). For example, *Zygosaccharomyces* and *Debaryomyces* were readily capable to grow at 55%. Other yeast species, such as *Starmerella* and *C. bombi* reached the same upper limit, but they showed a delayed growth response above 45-50% glucose. *Metschnikowia* strains mostly failed to grow at glucose concentrations above 50%. A classification of species based on their similarities in tolerance to high glucose concentrations did not reveal any distinct segregation of isolates from nectar and bee glossae (related to high glucose microenvironments) with respect to isolates from the phylloplane and the air (Table 2). However, highly

osmotolerant yeast species such as *Z. rouxii*, *D. hansenii*, *C. friedrichii* and *C. bombi* were found only among phylloplane isolates, and two of the three nectar yeast species did not grow in 40% of glucose medium (Fig. S1).

### *6. Growth rates and temperature responses*

As many as 38% of the species tested failed to grow during the first three days after plating, irrespective of temperature. Species with delayed growth responses comprised six *Cryptococcus* species (*C. festucosus*, *C. laurentii*, *C. magnus*, *C. oeirensis*, *C. stepposus*, *C. terreus*), along with the yeast-like fungus *Coniochaeta leucoplaca* and the plant pathogens *Dothiora elliptica* and *Dothichiza pythiophila.* Only 17% of the yeast species grew at temperatures below 12ºC within three days (Fig. 5). Specifically, *D. hansenii* and *C. friedrichii* had minimum growth temperatures of 8ºC and the three *Metschnikowia* species were the only isolates able to grow below this temperature. As to the upper limit of temperature tolerance, three species failed to grow at or above 30ºC (*C. friedrichii*, *C. victoriae*, and *D. nepalensis*). Only four species grow over the entire range observed in the field (8-30ºC). Those were *D. hansenii*, *M. gruessii*, *M. kunwiensis*, and *M. reukaufii.* In terms of cell density, five species reached the highest value of growth in this 3-day trial, namely, *C. bombi*, *D. hansenii*, *M. reukaufii*, and *O. zsoltii.*

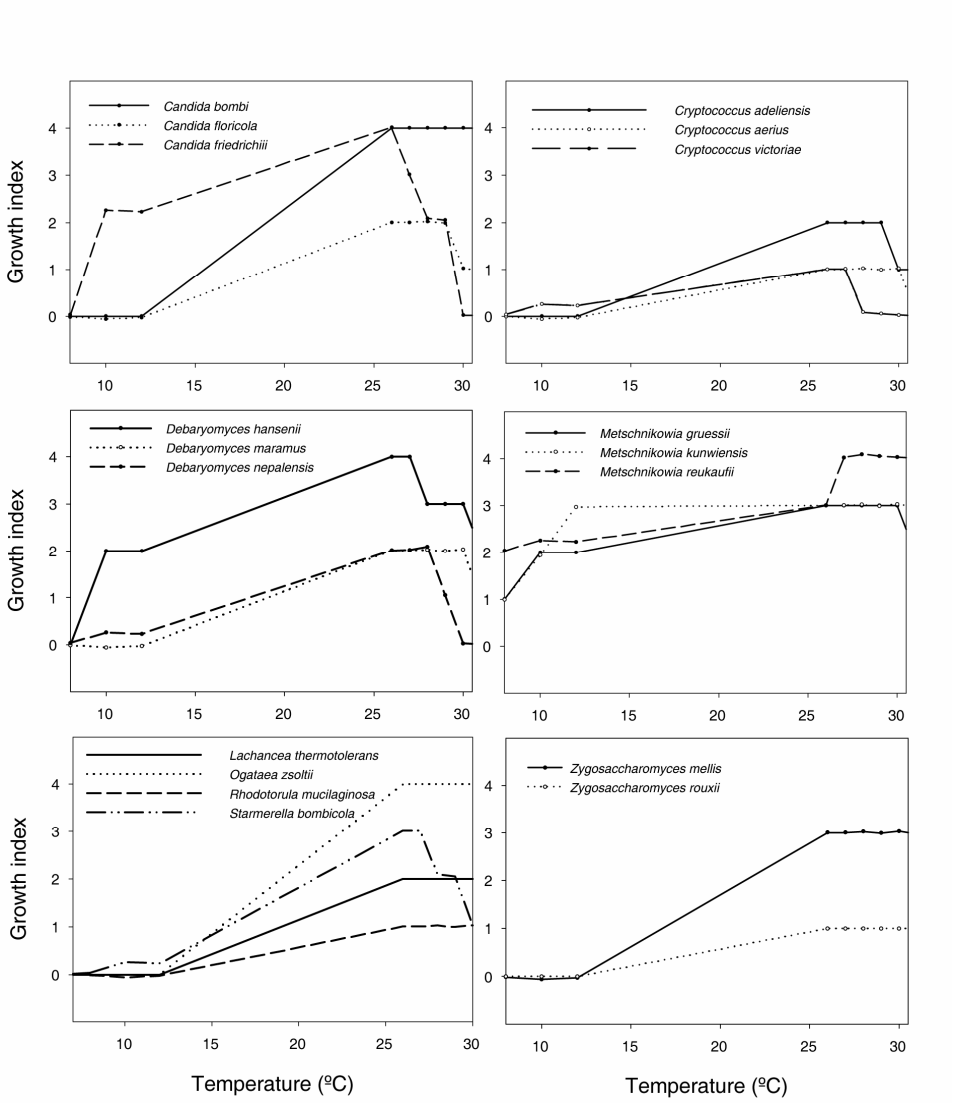


**Figure 5.** Growth curves of 29 yeast species each belonging to the following genera: *Candida* (a), *Cryptococcus* (b), *Debaryomyces* (c), *Metschnikowia* (d), Others (e) and *Zygosaccharomyces* (f) on YM medium at chamber temperatures from 8 to 30 °C.

## Discussion

Despite a substantial body of literature on air and insect-borne propagules and their significance to dispersal of epiphytic microbes (e.g., Andrews, 1991; Kinkel, 1997; Lachance *et al.*, 2001; Starmer *et al.*, 2003) there have been few

attempts to correlate quantitatively shifts in air and insect yeast inocula with specific changes in phylloplane and floral yeast communities (e.g., for air and phylloplane interchange, Andrews *et al.*. 1987). We found that external sources, namely atmosphere and flower visiting insects, were quantitatively important yeast donors to the phylloplane and flowers of the two plant species studied. Our results showed that nectar and pollen yeasts were mainly vectored by bees´ mouthparts, and only secondarily from the atmosphere.

The yeast assemblages recorded in this study could be divided into three main groups according to their microhabitats. Communities of mostly ascomycetous, osmotolerant species were associated with insect mouthparts *(i.e.*, *Debaryomyces, Metschnikowia*, *Starmerella,* or *Zygosaccharomyces* spp.), basidiomycetous (*Cryptococcus* spp.) yeasts were isolated primarily from phylloplane and air samples, and floral nectar harboured highly restricted communities, represented by *Metschnikowia gruessii* in *Digitalis* and by two fungal species in *Atropa,* composed by the basidiomycetous *R. mucilaginosa* and the ascomycete yeast-like fungus *C. leucoplaca*.

Yeasts from natural environments may be classified according to their growth characteristics (Davenport, 1975). In this study, yeast species associated with insect visitors and their floral rewards could be discerned from generalistic species from the phylloplane and the air on the basis of their carbon and nitrogen source utilization patterns. This division was largely due to the abundance of basidiomycetous yeast species on plant surfaces and the predominance of ascomycetous species in bees and nectar. Consistent with this distinction, the yeast communities of phylloplane and bee mouthparts were dissimilar. Typically insect-vectored species, like *Metschnikowia* spp., *Zygosaccharomyces* spp., and *Starmerella bombicola* (Rosa and Lachance, 1998; Rosa *et al.*, 2003), have been also isolated at a lesser frequency from corolla surfaces, which might be interpreted as a sign of niche specialization, but this also indicates that phylloplane strains cover a very broad range of physiological abilities. The phylloplane is generally considered oligotrophic (Andrews and Harris 2000), and depending on the system studied,

carbon compounds alone or both carbon and nitrogen compounds were shown to be limiting factors for bacterial and yeast populations on leaves (Bashi and Fokkema 1977; Mercier and Lindow 2000). From a microbe's perspective, the phylloplane is a continuously fluctuating physical environment, both spatially and temporally (Hirano and Upper 2000; Lindow and Brandl, 2003), and so a high physiological plasticity at the community level may be considered an indispensable step for its colonization.

Typical phylloplane species were isolated in the nectar of *Atropa*. In this plant species, nectar samples harboured yeasts only when insect visits were excluded, and so it is not surprising that nectar and bee glossae did not share any species in this case. The fungal species appearing in nectar (*R. mucilaginosa* and *C. leucoplaca*) have been previously isolated as endophytes (Unterseher *et al.*, 2010), suggesting that additional work should be done to assess the possibility that nectar yeasts in *Atropa* nectar may also originate from the plant tissues themselves. It is worth noting, however, that *R. mucilaginosa* is thought to be the most ubiquitous yeast known (Sampaio, 2010). Moreover, the two pigmented species found in *Atropa* nectar were isolated during summer months, when prevailing temperatures, moisture levels, day length, and intensity of sun exposure may be favouring those yeasts that presumably are better adapted: *C. leucoplaca* is a saprobic yeast-like fungus that is able to grow in dry conditions and whose maximum growth temperature is 34ºC (Cannon and Kirk, 2007, page 88) and *R. mucilaginosa* grows at temperatures up to 36ºC (data not shown).

The present study supports the conclusion that, in comparison with flower visiting bees and phylloplane, nectar has a notably lower yeast frequency and harbours a very limited set of yeast species. The low species richness of nectar yeast communities provides support for the "nectar-filtering hypothesis", as proposed in Herrera *et al.* (2010). According to this hypothesis, the high sugar content and the presence of plant secondary compounds that usually characterizes nectar samples, as was noted in the introduction, may have a strong impact on the survival of incoming yeasts. The main nectarivorous *Metschnikowia* species, as described by this

study and previous work conducted in the same region (Herrera *et al.*, 2009, Herrera *et al.*, 2010, Herrera and Pozo, 2010, Pozo *et al.*, 2011) may then possess some suite of specific physiological traits which allow them to successfully overcome nectar filtering.

***Secondary compounds and sugars***

The specific resistance of members of the Metschnikowiaceae clade to plant secondary compounds and high glucose concentrations will be discussed below, by comparing nectar-inhabiting species with the potential pool of species arriving to floral nectar in the two plant populations surveyed. In our attempt to detect specific resistance of nectar yeasts to toxic compounds produced by the plant species examined in this study, *Digistalis* cardenolides did not show any inhibitory effect on yeast growth, and *Atropa* alkaloids were equally toxic to all yeast colonizers. Given that we tested concentrations of these compounds that go well beyond the proposed threshold for plant parts of *Atropa sp.* (Zárate *et al.*, 1997), the inhibitory effect of alkaloids may have been overestimated. We can certainly conclude that nectarivorous yeast species did not show any advantage with respect to resistance to secondary compounds, as it was pointed out by Manson *et al.* (2007). Despite the lack of inhibitory effect of glycosides on yeast growth in our assays, it cannot be ruled out that a complex mixture of secondary compounds and primary metabolites, or even a mixture of these substances and inorganic ions could be effective against yeasts. It is also possible that the commercial substances used in those experiments were slightly different from those occurring in nature. All the same, both plant species harboured yeasts in their nectar samples, such that we cannot conclude that the plant secondary compounds used in our assay were inhibitors in natural conditions for nectar-depleting yeasts, as it has been proposed by the "antimicrobial hypothesis" (Adler, 2000; Golonka, 2002, *as cited in* Antonovics, 2004; Irwin *et al.*, 2004; Manson *et al.*, 2007).

The principal factor thought to limit nectar community composition is the inability of a high proportion of colonizers to survive the high sugar conditions of nectar. *Atropa* nectar is characterized by a very high sugar concentration (% of total

sugars, w/w, mean ± SD; 46.5 ± 22.1) and *Digitalis* nectar possess more moderate sugar concentration (16.2 ± 2.9). In spite of this, most yeast species found to be osmophilic were not recovered from nectar samples from either of the two plant species surveyed. The exception was *Metschnikowia gruessii*, which is able to grow in the presence of up to 50% glucose and was found in nectar samples examined in our study.

High sugar content and plant secondary compounds alone thus do not explain the prevalence of *Metschnikowia* species in floral nectar, which raises the question of what additional factors should be considered in the "nectar-filtering" hypothesis. The possibility cannot be ruled out, however, that some of our negative results were the consequence of insufficient statistical power due to relatively small sample sizes (i.e., committing Type II error, or failing to reject a false null hypothesis). The information available only allow to speculate that, if such effects do actually exist, they probably are neither strong nor pervasive.

Recent studies have suggested that interspecific differences in thermal tolerance limits or growth responses can play a central role in yeast community organization by influencing the species composition of communities (Lachance *et al.*, 2003, Sweeney, 2004; Goddard, 2008, Sampaio and Gonçalves, 2008). Differential thermal tolerance may determine what yeast species survive in natural populations for a given season, but this does not explain why the lowest species richness was in fact found in nectar samples. Moreover, draining and direct radiation may strengthen this effect on the phylloplane.

***Yeast species growth rate***

Rapid growth should be a significant advantage for nectar colonizers, given the ephemeral nature of this resource. Nectar secretion by individual flowers at the two spring-summer blooming plant species examined in this study lasts for up to three days. Yeast species with a delayed growth response, in such a short timespan, should be "swept" by competition from the faster-growing *Metschnikowia* species. Even at low temperatures, *Metschnikowia* species reach a high growth rate, higher than that of other specie which is capable to grow between 8 and 31ºC within 3

days, such as *Debaryomyces hansenii*. The temporal constraints imposed by the short duration of the floral microhabitat could also explain the lower frequency of yeasts in nectar samples compared to external sources and the phylloplane. Assuming that the ephemeral nature of flowers and/or the nectar secretion phase may be a strong limiting factor for nectar-colonizing yeasts, this duration-restricted effect may be strengthened in plant species with high visitation rates. In *Digitalis* plants, four species of social bees were the main visitors during our experiment. In the *Atropa* population, we counted more insect visits and visitors covered a broader spectrum that included solitary bees, social bees, and Lepidoptera. When we explored pollinator composition and nectar yeast frequency and species richness in a broader range of plant species and study sites in the same region (one population each of 13 plant species, MI Pozo and CM Herrera, *unpublished results*), the frequency of total flower visits by social bees was correlated positively with yeast frequencies in the nectar samples ($r_s=0.55$, $p=0.05$). In contrast, the frequency of visits by solitary bees correlated positively with nectar yeast species richness (13 populations, $r_s=0.65$; $p=0.01$).

In conclusion, results of this study strongly suggest a combined influence of immigration history and different ecological and selective forces operating at each microhabitat as the driving features ultimately determining yeast communities, in terms of both yeast incidence and yeast species composition. Additional work is needed to untangle the complex combination of suggested factors here, and the main conclusions of this study should be tested under changing environmental conditions and for different host plants.

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## Supplementary material

**Table S1.** Number of visits and relative abundance of the different flower visiting insect taxa in the censuses in each of the two plant species studied.

| Insect visitor | | Plant specie | | | |
|---|---|---|---|---|---|
| | | *Digitalis obscura* (26) | | *Atropa baetica* (26) | |
| Species | Family | Abundance (indiv/cont) | Visit rate (flowers/ min) | Abundance (indiv/cont) | Visit rate (flowers/ min) |
| Bees: | | | | | |
| *Anthophora (Amegilla) quadrifasciata* | Anthophoridae | - | - | 0.04 | 5.45 |
| *Apis mellifera* | Apidae | 0.04 | 8 | | |
| *Bombus pascuorum* | Apidae | 0.23 | 5.25 | 0.08 | 2.61 |
| *Bombus pratorum* | Apidae | 0.85 | 5.40 | - | - |
| *Bombus terrestris* | Apidae | 0.08 | 4.23 | 0.65 | 4.26 |
| *Lasioglossum spp.* | Halictidae | - | - | 0.69 | 3.92 |
| *Xylocopa violacea* | Anthophoridae | - | - | 0.04 | 3.64 |
| Butterflies: | | | | | |
| *Gonepteryx cleopatra* | Pieridae | - | - | 0.04 | 2.22 |

Values in brackets are number of 3 min. censuses.

**Table S2**. Factor loadings for the first two PCs based on 29 yeast species growth response (0-4 index) to 82 different tests.

| Test | PC1 loadings | PC2 loadings |
|---|---|---|
| L-arabinose | 0.80 | 0.45 |
| 2-KetoGluconate | 0.76 | -0.20 |
| Inositol | 0.73 | 0.48 |
| Raffinose | 0.73 | 0.13 |
| Gluconic acid | 0.70 | -0.37 |
| Trehalose | 0.69 | -0.15 |
| Cyclohexamide 10 ppm | 0.69 | 0.02 |
| Rhamnose | 0.68 | 0.20 |
| Xylose | 0.68 | 0.22 |
| Cyclohexamide 100 ppm | 0.66 | -0.09 |
| Salicin | 0.66 | -0.33 |
| D-Glucuronate | 0.65 | 0.43 |
| Ribitol | 0.64 | -0.53 |
| Mannitol | 0.63 | -0.44 |
| Digitonin 8 ppm | 0.63 | -0.09 |
| Lactose | 0.62 | 0.23 |
| Cellobiose | 0.61 | -0.37 |
| α-Methyl-glucoside | 0.61 | -0.43 |
| Sodium nitrite | 0.61 | 0.38 |
| Succinic acid | 0.59 | -0.01 |
| N-acetylglucosamine acid | 0.58 | -0.54 |
| Galactose | 0.57 | -0.43 |
| Galactitol | 0.57 | 0.25 |
| Lipids | 0.56 | 0.35 |
| Maltose | 0.55 | -0.39 |
| Melibiose | 0.55 | 0.26 |
| Erythritol | 0.51 | -0.14 |
| Ribose | 0.50 | -0.02 |
| YM 6 °C | 0.49 | 0.07 |
| Glucose | 0.49 | -0.52 |
| YM 8 °C | 0.48 | -0.16 |
| Sodium nitrate | 0.47 | 0.55 |
| 5-Fluorocytosine | 0.46 | 0.29 |
| YM 4 °C | 0.46 | -0.25 |
| Ym 10 °C | 0.41 | -0.14 |
| Glucitol | 0.40 | -0.11 |
| Xylitol | 0.40 | -0.52 |
| Glucosamine | 0.40 | 0.04 |
| Phenylalanine | 0.39 | 0.31 |
| D-arabinose | 0.37 | 0.20 |
| NaCl 15% | 0.36 | -0.40 |
| Inulin | 0.36 | 0.01 |
| Vitamin free | 0.36 | -0.25 |
| Sucrose | 0.34 | -0.25 |
| YM 12°C | 0.33 | -0.21 |
| Ethanol | 0.32 | -0.53 |
| YNB-G 4 °C | 0.31 | -0.41 |
| Malic acid | 0.28 | -0.17 |
| n-Butanol | 0.28 | 0.06 |
| Ethyl-acetate | 0.27 | -0.09 |
| Sorbose | 0.21 | -0.62 |
| Melezitose | 0.19 | -0.37 |
| Canavanine | 0.16 | -0.15 |
| NaCl 5% | 0.16 | -0.62 |
| Glycerol | 0.14 | -0.64 |
| NaCl 10% | 0.12 | -0.78 |
| Gelatin | 0.10 | 0.12 |
| Casein | 0.09 | -0.18 |
| Sulfuric acid | 0.05 | -0.17 |
| Gluconolactone | 0.04 | -0.28 |
| CTAB 10 ppm | 0.00 | -0.65 |
| Citric acid | -0.05 | -0.02 |
| Taninin | -0.12 | -0.52 |
| Ethylamine | -0.15 | -0.65 |
| Hexadecane | -0.16 | -0.59 |
| Sodium carbonate | -0.16 | -0.41 |
| Deoxycholate | -0.16 | -0.72 |
| YM 33 °C | -0.17 | -0.49 |
| Lysine | -0.23 | -0.67 |
| Glucose 50% | -0.26 | -0.50 |
| YM 36 °C | -0.26 | -0.17 |
| YM 34 °C | -0.26 | -0.19 |
| YM 35 °C | -0.29 | -0.21 |
| YM 32 °C | -0.29 | -0.59 |
| Ethanol 6% | -0.35 | -0.26 |
| Cadaverine | -0.39 | -0.60 |
| YNB-G 30 °C | -0.44 | -0.50 |
| YM 28 °C | -0.51 | -0.41 |
| YM 29 °C | -0.52 | -0.44 |
| YM 31 °C | -0.54 | -0.47 |
| YM 30 °C | -0.61 | -0.43 |
| Glucose fermentation | -0.66 | -0.46 |

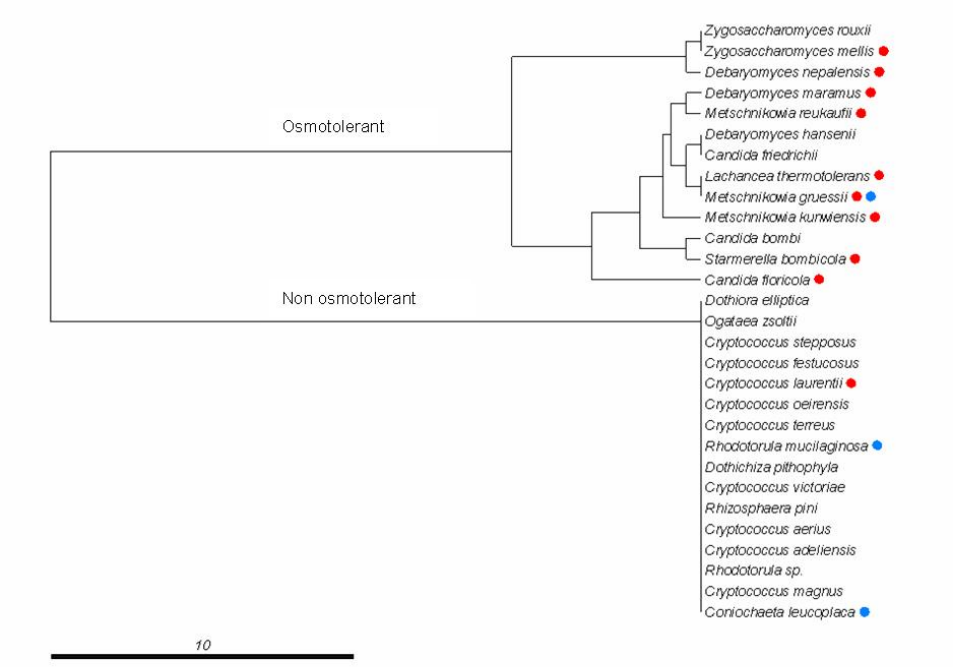
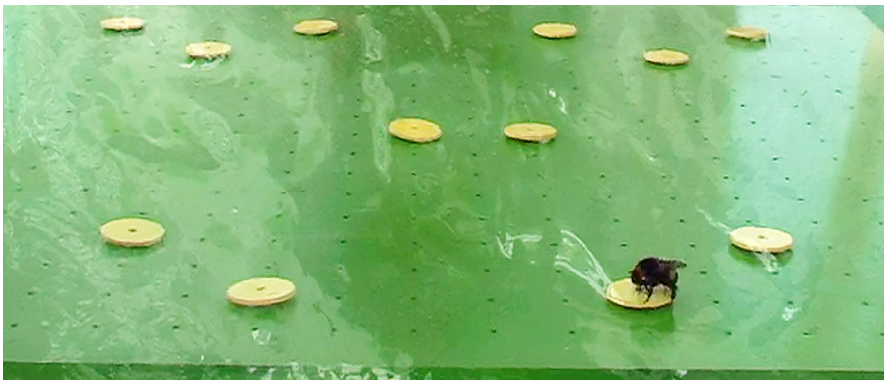



**Figure S1.** Dendrogram depicting the similarity between 29 yeast species isolated from two localities in SE Spain according to growth intensity in media with increasing glucose concentrations. Solid dots are used for species which have been isolated from microsites related to high sucrose concentration, i.e., nectar (blue) and bee glossae (red). Scale bar length indicates dissimilarity among taxa based on Euclidean distance matrix.

# Yeasts as modifiers of floral nectar: effects on bumblebee foraging.



# CAPÍTULO 5

*Las levaduras como agentes modificadores del néctar floral: su efecto sobre el forrajeo de los abejorros.*

# CAPÍTULO 5

## Yeasts as modifiers of floral nectar: effects on bumblebee foraging

*Las levaduras como agentes modificadores del néctar floral: su efecto sobre el forrajeo de los abejorros.*


## Abstract

Yeasts occur regularly in floral nectar, where they produce density-dependent modifications in nectar quality, such as sugar concentration decline or change in sugar composition. Although these modifications, along with the yeast presence in floral nectar itself, are supposed to impact pollinator choice, these empirical connections still await confirmation. The purpose of this paper is to describe, by conducting controlled experiments, the effect of nectar inhabiting yeasts on pollinator foraging behavior. We will assess the ability of naïve bumblebees (captive colonies of *Bombus terrestris*) to detect and react to yeast presence in sugary solutions contained in artificial flowers, along with their detailed response to waning in total sugar concentration, changes in sugar composition and raise of among flowers variance in reward value, as produced by nectarivorous yeast in natural habitats. Bumblebee preferences were tested by two-treatment choice experiments, by comparing number of visits and probing time among treatments. We demonstrated that bumblebees can discriminate artificial nectars inoculated with yeasts, and that they were attracted to them. Bumblebees were highly sensitive to sugar concentration decrease, but they did not show any foraging preference for sample heterogeneity or different sugars. Our results call for a more comprehensive study of the role of yeast inhabiting floral nectar on their host plants through insect pollinator behavior in their natural habitat.

## Resumen

Las levaduras habitan con frecuencia el néctar floral, donde producen, en función de su densidad, una serie de modificaciones en la calidad del néctar, como la disminución en el contenido de azúcares o modificaciones en la composición de azúcares. Aunque estas modificaciones, junto con la propia presencia de las levaduras, están llamadas a incidir en la elección de los polinizadores, esta conexión empírica está aún por establecerse. El objetivo de este trabajo es describir el efecto de las levaduras habitantes del néctar sobre el comportamiento de forrajeo de los polinizadores, mediante la realización de experimentos en condiciones controladas. Se valoraron la capacidad de detección y reacción de abejorros sin experiencia en el forrajeo (colmenas cautivas de *Bombus terrestris*) ante la presencia de levaduras en flores artificiales con soluciones azucaradas, así como la respuesta detallada de estos insectos a la disminución en la concentración de azúcares, cambios en la composición de azúcares e incremento en la varianza entre flores en cuanto al valor de la recompensa floral, en situaciones que simulan los efectos de las levaduras en condiciones naturales. Se analizaron las preferencias de los abejorros mediante experimentos de elección entre tratamientos pareados, comparando el número de visitas y la longitud del tiempo de ingesta entre los dos tratamientos ofrecidos simultáneamente. Los abejorros discriminaron la presencia de las levaduras en las flores artificiales, y mostraron una mayor atracción por este grupo. Los insectos también respondieron significativamente a una disminución en la concentración de azúcares, pero no mostraron ninguna preferencia en los tests de heterogeneidad en la recompensa o ante distintos azúcares. Estos resultados animan a la realización de estudios más detallados y en condiciones naturales que permitan evaluar el papel de las levaduras en el forrajeo de los polinizadores, y consiguientemente, en el éxito reproductivo de las plantas hospedadoras.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## Introduction

Insect pollinators collect floral rewards such as pollen and nectar during foraging, and their behaviour ultimately promotes sexual reproduction in plants. In order to influence certain aspects of pollinator behavior, as frequency of visits, number of flowers probed per plant, probing time per flower, or even movement after leaving a given plant, plants correspondingly evolve subtle strategies, many of which are associated with "fine-tune" variation of the most specific reward nectar resources (Rathcke, 1992).

Furthermore, the pollinators themselves can promote important modifications in nectar characteristics by vectoring micro-organisms such as yeasts into floral nectar (Canto *et al.* 2008). After initial colonization, yeasts become extremely abundant in the nectar of many plants (Brysch-Herzberg, 2004; de Vega *et al.*, 2009; Herrera *et al.*, 2009). When yeasts are present in floral nectar, they cause a density-dependent decline in total sugar concentration (Herrera *et al.*, 2008, de Vega *et al.*, 2009). Nectarivorous yeasts also induce changes in nectar sugar composition, such as an increase in fructose content in detriment of sucrose (Herrera *et al.*, 2008). Yeast distribution in nectar samples may be extremely patchy for a single host plant, with variation in yeast density mostly occurring at the within-plant level (Pozo *et al.*, 2009). As a result, flowers in the same plant may have variable yeast densities in nectar, thus giving rise to a variable distribution of reward at the within-plant level (Herrera *et al.*, 2008). Sensorial cues, as nectary content warming, have been recently correlated to yeast metabolic activity (Herrera and Pozo, 2010), and flower odor may be modified by the emission of yeast fermentation products (Raguso, 2004; Goodrich *et al.*, 2006). Yeast-induced variation in sugar content, nectar temperature or odor can also influence pollinator attraction and behavior (Raguso, 2004; Dyer *et al.*, 2006; Whitney *et al.*, 2008).

Yeasts presence in floral nectar may thus have broad ecological implications through its effects on pollinators, and several studies have mentioned the relevance of explicitly including nectarivorous yeasts in the study of plant-pollinators relationships (e.g., Kevan *et al.*, 1988; Eisikowitch *et al.*, 1990; Canto *et*

*al.*, 2008; de Vega *et al.*, 2009; Herrera *et al.*, 2009; Pozo *et al.*, 2009). The strength of these tripartite interaction links, however, depends on the extent of pollinators foraging behaviour disruption caused by the presence of yeasts in floral nectar. As far as we know, there is just one study focusing specifically on the ability of pollinators to respond to yeast presence, and it showed that honeybees did not discriminate yeasts presence in nectar experimental units (Kevan *et al.* 1988). Nevertheless, volatile emissions by certain yeasts species are attractant to beetles (Graham *et al.*, 2011), and some plants species mimic yeasty fermentation odors to increase their attractiveness to pollinators (Goodrich *et al.*, 2006; Stökl *et al.*, 2010).

Pollinator response to yeast contaminated nectar may be still broad enough to include more than discrimination of the yeast cells presence itself or yeast metabolites. Yeast-contaminated nectar, as it was noted above, also includes a "chemical signature", mainly characterized by sugar concentration decline, change in sugar composition, and increment of the within-plant reward variance (Herrera *et al.*, 2008). Energetically-constrained pollinators are expected to be sensitive and to respond to these modifications in nectar quality and reward distribution derived from nectarivorous microbes' activity.

The objective of this paper is to explore experimentally the possible effects of yeast presence in floral nectar on pollinator foraging behavior. Our model organism will be a bumblebee, *Bombus terrestris* (L.), whose ability to fly in cool weather renders them one of the most important pollinators in cool and temperate regions. We will adopt two complementary approaches. In the first one, we will directly assess the ability of naïve bumblebees to detect and react to yeast presence in sugary solutions contained in artificial flowers. In a second step, we will mimic separately three main effects that are usually produced by yeast metabolism in floral nectar, as i) reduction in total sugar concentration ii) changes in sugar composition and iii) increased among-flowers variance in per-flower energetic reward, measuring bumblebees response to each of these effect. Our results provide novel evidence suggesting that bumblebees can detect and respond positively to nectarivorous

yeasts, and this call for a more comprehensive study of the role of yeast inhabiting floral nectar on their host plants via their effects on insect pollinators.

## Materials and Methods

All experiments were conducted by using captive *Bombus terrestris* colonies (Koppert Biological Systems) housed in plastic nest boxes. Inside the hive, pollen was offered in one feeder, and sugar solution was supplied in a bag below the nest, set up so as to permit the solution to drip. In order to maintain bees in a reasonable sugar-limited state before every experimental run, we kept the bees starved for 24 h before trials, by using alternatively two colonies of similar age. Experimental bees had never previously foraged on nectar from natural flowers, and were naïve with respect to the experimental setup before training began. Experiments were conducted in spring 2010 (from 8 April to 21 May) and 2011 (from 16 March to 8 April) in a greenhouse. Greenhouse temperature was recorded every 30 minutes during the experimental period by using a Tinytag datalogger (Gemini Ltd., UK) placed inside the greenhouse. Averaged daily temperatures during the experimental period were (mean ± SD) 22.0 ± 2.8 ºC and 20.8 ± 2.6 ºC in 2010 and 2011, respectively. An averaged daily air relative humidity of 55.1 ± 12.6 was measured in 2010, and 61.8 ± 11.0% in 2011.

The bee hive was connected to a flight cage (60 x 60 x 60 cm; front and back in clear vinyl, right and left in polyester netting mesh, Bugdorm, Megaview Ltd.) by means of a plastic transparent pipe, regulated by two serial gates. A 6 mm green Plexiglas sheet was used as a "bee board" (42 x 42 cm) and was placed inside the flight cage. Wells 3 mm deep and 2 cm apart were drilled into the bee board, generating a uniform distribution of 324 wells. Twelve wells were covered with yellow painted 1 mm deep Plexiglas round pieces of 2 cm diameter with a central hole of 1 mm to generate artificial flowers. As the experiments included in this study were ultimately designed to simulate the characteristics and arrangement of yeast-containing flowers in a natural scenario, each experimental group in the bee board simulates an inflorescence, by symmetrically placing flowers in four

inflorescences by groups of three, as indicated in Fig. 1. Before every experimental run, each individual forager was allowed to access the flight cage and stay for 30 minutes in contact with the artificial flowers. During the training phase the artificial flowers were filled with 6 μL of the "learning solutions". Different learning solutions were selected for each experiment, as they should differ to the experimental ones. Once bumblebees had probed the flowers, flowers were depleted and cleaned with ethanol 20% solution to remove scent marks. All the other intermediate wells remained empty during training and experimental phases. The successfully trained individuals were marked by gluing numbered tags to their backs.

In order to avoid artifacts due to spatial preferences of the individual foragers, each experimental group was replicated twice in the bee board, by placing the same reward type (treatment 1) in A and D groups, and the alternative reward type (treatment 2) in groups B and C; and each bee was tested twice without leaving the cage, alternating the location of each reward type between trials (first and second phase). Each experimental flower contained 3 μl of the experimental feeding solution, so total "nectar" availability was 36 μl (*N*= 4 groups x 3 flowers/group x 3 μl per flower) at each experimental run. Between experimental runs, all the flowers were depleted and cleaned with a 20% ethanol solution.

Experiment 1 was designed to test the effect of yeast presence, itself, on bumblebees foraging. Experiments 2 and 3 simulated the effect that nectarivorous yeasts produce on nectar sugar concentration and sugar composition, respectively. Experiment 4 simulated a randomly varying nectar environment within inflorescences such as those created by patchy within-plant yeast distributions, which in turn produce a patchy distribution of nectar concentration. Solute concentrations will be given in a weight to weight percent basis.

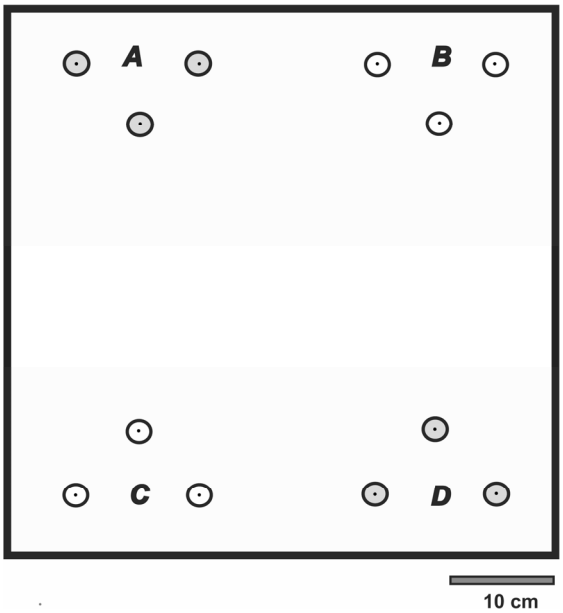


**Figure 1.** Schematic drawing of the artificial flowers (circles) arrangement by experimental groups (letters A-D) in the bee board. Groups belonging to the same experimental treatment (1 or 2) share circles fill colour.

***Experiment 1, "Yeast presence"***

We prepared liquid yeast suspensions in sugar broth base (1% yeast extract, 1% peptone, 1% glucose), adjusting a final density of 25000 cells $mm^{-3}$. Three yeast species were used separately in experiments, namely *Metschnikowia reukaufii*, *Metschnikowia gruessii* and *Candida bombi,* all of which are frequently found in the nectar of bumblebee pollinated plants in western Europe (Brysch-Herzberg, 2004; Herrera *et al.*, 2010; Pozo *et al.*, 2011). We used one strain per specie, all of which came from the laboratory culture collection, comprising field-collected nectar yeast isolates. Yeast cultures were kept refrigerated until utilization in experiments, which was done within 24h.

Artificial flowers in the experimental group with nectar supplemented with yeasts contained 1 μl of yeast suspension in sugar broth base plus 2 μl of sucrose 35%, mixing both solutions immediately before trials. Control flowers were filled

with 1 μl of sterile yeast broth base plus 2 μl of 35% sucrose. Training phase was conducted by using sucrose 30%.

***Experiment 2, "High Vs Low rewarding"***

We prepared sucrose solutions of 50, 25 and 12.5%, diluted in sterilized water. These sugar concentrations encompass most of the range naturally occurring in the nectar of the yeasts' host plants in a Mediterranean mountainous plant community (Herrera *et al.*, in press Mol Ecol DNA methylation). We assessed sugar concentration decline effect on bees by simultaneously offering bumblebees sucrose solutions of 50 and 25% ("high vs. low"); or 50 and 12.5% in the "high vs. very low" sugar concentration comparisons. Training phase was conducted by using sucrose 30%.

***Experiment 3, "Sugar type"***

On each trial bumblebees were offered paired combinations of fructose (F), glucose (G), and sucrose (S) plain solutions. Sugar concentration was standardized within trials, and two series of experiments were conducted, by comparing sugar preferences (F-G; F-S; G-S) at total sugar concentration of 25 and 50%. A mixed solution, combining equal volumes of each of the two sugar solutions involved in the corresponding experimental run, was used in the training phase.

***Experiment 4, "Variable vs. Constant reward"***

Bumblebees were offered alternative group-variance treatments with mean sucrose concentration of 15%. Specifically, within group variable nectar concentration was simulated in one treatment by filling the three flowers of each group with 0, 15 and 30% sucrose solutions, respectively. In contrast, in the constant group treatment all flowers were filled with a 15% sucrose solution. Training phase was conducted by using sucrose 10%.

***Data collection and statistical analyses***

Bumblebee behaviour during experimental runs was videorecorded with a HDMI videocamera (Sony HDR-SR7) placed in front of one side of the flight cage. Recording began after introducing each outgoing bee, and lasted for 15-60 minutes, according to the bee activity on the experimental flowers. Images were analyzed using Picture Motion Browser (Sony). Bumblebees often hovered in front of flowers, sometimes briefly touching the artificial flower disk and then departing without actually making any attempt at probing nectar. Videorecorded sessions allowed to distinguish these rejected flowers from effective visits in which nectar was effectively probed. From those effective visits we recorded the duration in seconds during which the focal bee was licking. Total probing time and number of visits varied widely among experimental runs, so number of acceptances and feeding period were transformed into bee preferences by dividing the number of visits and the probing time in each alternative treatment by the total number of visits and feeding time, respectively, along each experimental run. A total of 20 bees were successfully trained for each experimental category, but bee recordings were discarded if bees did not probe the flowers during the experimental run, or if they had probed only one of the two choices (7% of the total bee recordings). This criterion was introduced in order to obtain more conservative statistical estimates, and we consistently obtained that, in trials in which just one of the two choices were probed, bee responses did not differ to those expected just by chance. As a result, we analyzed a total of 28 bees for experiment 1, which were exposed either to *M. reukaufii* (11), *M. gruessii* (11) and *C. bombi* cells (6). Fifteen bees were used in experiment 2 analyses, where seven and eight bees belonged to "high vs. low" and "high vs. very low" sugar concentration preference tests, respectively.A total of 31 bees were available for experiment 3 analyses, five bees corresponded with sugar preference tests performed at 25% and 26 bees to those conducted at 50% total sugar concentration. Finally, 13 bees were finally used in experiment 4 analyses.

All statistical analyses were conducted using the SAS statistical package (Cary, USA). For each experiment, relative probing time and relative number of

effective visits were the two response variables analysed with a mixed model ANOVA (Proc Mixed). For experiment 4, we also analyze the minimum residence time within a group of flowers before departing to another "inflorescence" as a response variable. In all cases treatments were treated as fixed factors and individual bee and experimental run were introduced as random effects. Least-squares means ± SE, that estimate the marginal means corresponding to the fixed effects over a balanced population, were given herefter to characterize bees response, and these values will be shown in proportion.

In Experiment 1, we first considered "yeast presence" irrespective of the species assayed, as a fixed factor with "presence" and "absence" as the two experimental levels. In a second step, we dissected the effect according to species identity, thus considering the following four treatments levels, "without yeasts", "*M. reukaufii*", "*M. gruessii*", and "*C. bombi*". In experiment 2, the fixed factor was "sugar concentration", with three different levels, 12.5% as "very low", 25% as "low" and 50% as "high" rewarding categories. In experiment 3, we analysed the effect of two fixed factors, "sugar type" (with fructose, glucose and sucrose as levels), and "sugar concentration" with two levels, 25 and 50%, and the interaction between sugar type and concentration. In experiment 4, we considered "within-group variance" as fixed factor and "variable" and "constant" as levels.

We conducted "post hoc" analyses (Tukey`s HSD pairwise comparisons of model-adjusted least squares means) to determine the levels in which the significant differences were driven.

We confirmed the reproducibility of our results by considering, along with the corresponding fixed factor in each experiment, the experimental phase (first or second trial per bee in a given experimental setup) and the interaction of both factors on the foraging preferences of individual bumblebees. We consistently found that foragers showed the same preferences irrespective of the experimental phase (results not shown). However, behavioral response variance among experimental runs per bee and among individual bees was considered in analyses by introducing them as random effects, as it was noted above.

## Results

### *1. Effect of adding yeasts to artificial nectar*

Bumblebees showed a clear preference for artificial flowers in which yeasts had been added to nectar. Experimental bees expent significantly more time probing nectar from yeast-inoculated flowers (relative probing time= 0.55 ± 0.03) than from those in the control group (0.44 ± 0.03; $F_{1,43}$= 6.50, p= 0.0133). They also showed their preference for yeast-inoculated nectar in the lower proportion of visits to yeast-free (0.42 ± 0.03) as compared to yeast-contaminated flowers (0.58 ± 0.03; $F_{1,43}$= 16.58, p= 0.0002).

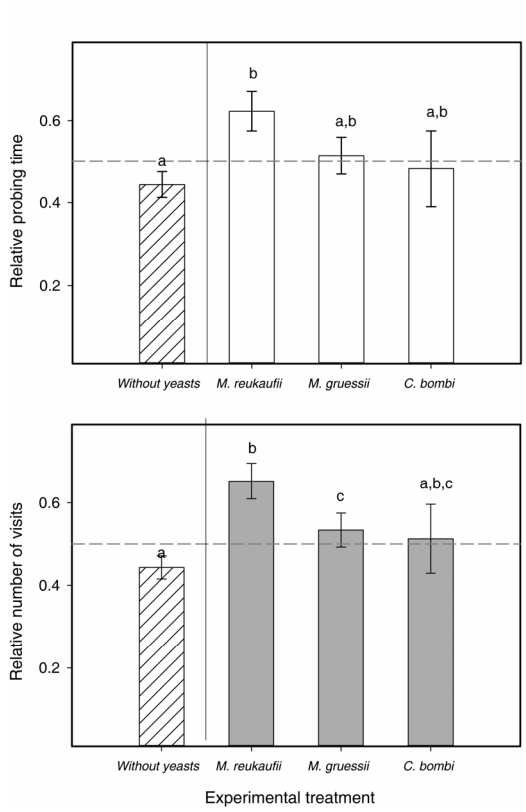


**Figure 2.** Bee preference for yeast-free or yeast-inoculated nectar using three different species of floral nectar yeasts, *M. reukaufii*, *M. gruessii* and *C. bombi*, judged by the relative probing time (upper panel) and relative number of visits (bottom panel) to each treatment. Each yeast species was always compared to the control yeast-free nectar (see Results for details) and dashed line at 0.50 indicated absence of preference. Different letters above bars denote a statistically significant difference among mean values ($p < 0.05$).

There were also differences among yeast species in the magnitude of the bee response (Fig. 2). Bee relative probing time compared to control yeast-free nectar was only significantly higher in flowers with *M. reukaufii* (Fig 2, upper panel). Concerning the proportion of flower visits, adding *M. reukaufii* and *M. gruessii* cells to artificial nectar induced a statistically significant attractant effect on foraging bees, with a stronger response in *M. reukaufii* (Fig. 2, lower panel).

***2. Effect of sugar concentration***

Bumblebees showed a clear preference for more concentrated nectar (Fig 3). There was a significant overall effect of sugar concentration on bee preference in terms of relative number of visits ($F_{2,24}$= 3.40; p= 0.05). Post-hoc comparisons revealed that this effect was mostly due to a significant difference between 50 and 12.5% sucrose treatments (t= 2.32; p= 0.03).

Consistently, bumblebees probed flowers containing sucrose 50% for a longer period in comparison with those containing sucrose concentrations of 25 and 12.5% (Fig. 3), although those differences did not reach statistical significance ($F_{2,24}$= 2.57; p= 0.09).

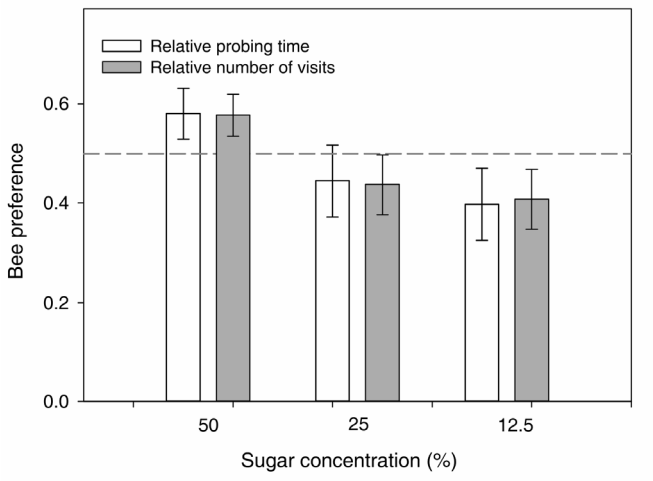


**Figure 3.** Bee relative probing time (white bars) and relative number of visits (grey bars) in response to sucrose concentration decrease. The dashed line at 0.50 indicated absence of preference.

### *3. Effect of sugar composition*

Bumblebees did not show a clear preference for any sugar in terms of number of visits or probing time. The number of visits performed to each of the three sugar solutions was nearly constant, especially at a sugar concentration of 25% (Fig. 4). However, we found an interaction between sugar type and concentration in the relative probing time ($F_{2,53}$= 1.78; p= 0.17). Whereas bees preferred the two hexoses (fructose and glucose) over sucrose at sugar concentration of 25%, they preferred sucrose over fructose and glucose at a total sugar concentration of 50%.

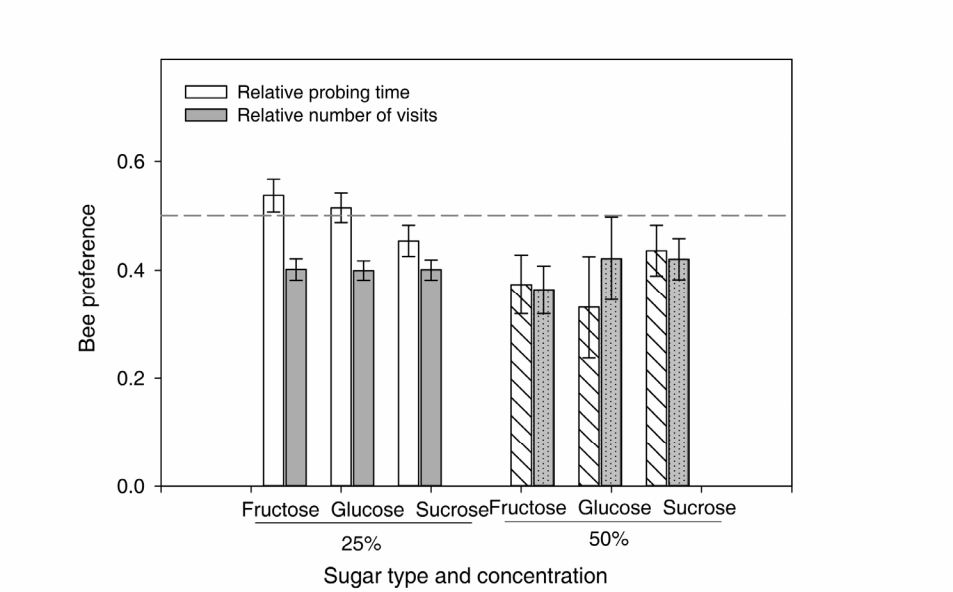


**Figure 4.** Bee response to alternative sugar types at total sugar concentration of 25 (solid color bars) and 50% (patterned bars). Bee preferences for fructose, glucose and sucrose nectars were computed as relative probing time (white bars) and relative number of visits (grey bars) to each treatment after three possible paired sugar-type comparisons within each concentration (see Methods for details). The dashed line at 0.50 indicated absence of preference.

### *4. Effect of variance in sugar concentration*

Bumblebees did not exhibit differences in probing time or number of visits between groups with the same mean sugar concentration but different within-group variance, yet there was a trend towards performing more visits to constant groups (Fig. 5, upper panel). Minimum residence time within flower groups was longer in the constant treatment than in the variable one (Fig. 5, lower panel, $F_{1,24}$= 1.93; p= 0.17), thus suggesting that the probability of early bumblebee departure from a patch increased in a context of variable distribution of rewards.

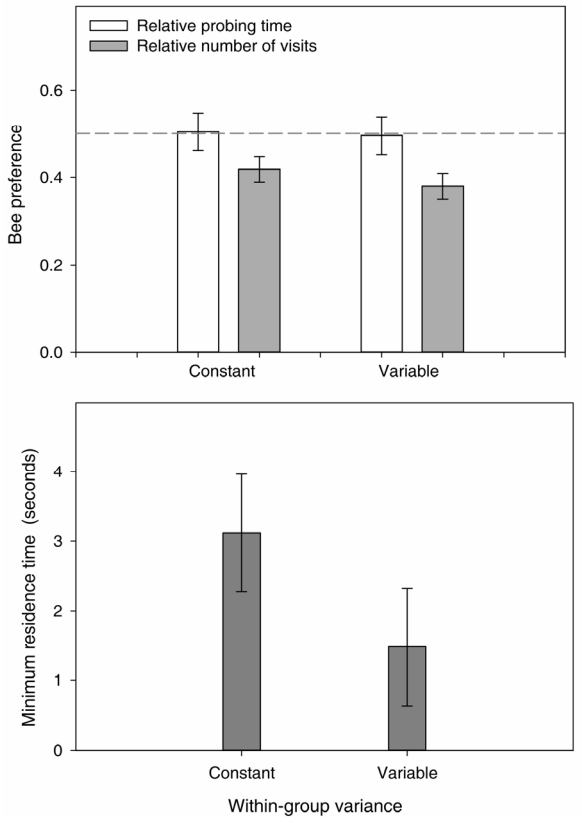


**Figure 5.** Bee preferences in response to constant and variable distribution of nectar reward within experimental groups. Upper panel, relative probing time (white bars) and relative number of visits (grey bars); lower panel within group minimum residence time.

## Discussion

We have shown that, under controlled experimental conditions, *B. terrestris* individuals detected the presence of yeasts in artificial sugar solutions mimicking floral nectar and responded positively to them. The magnitude of the response depended on the yeast species involved, and bumblebees were especially attracted by the addition of the most frequent and widespread nectar-living yeast *M. reukaufii*. Among the other changes that yeast metabolism is able to induce on nectar (sugar concentration, sugar composition, and heterogeneous distribution of reward), bees just showed a clear negative response to reduction of sugar concentration. In the following paragraphs we will discuss the soundness of these results in the context of plant-pollinator interactions.

### *Bumblebee response to yeast presence*

The effect of yeast-contaminated nectar on pollinators foraging under controlled conditions has been rarely studied before and never specifically with bumblebees. Nectar yeast detection by honeybees was previously examined by Kevan *et al.* (1988), by performing two series of experiments, (i) comparing excluded and open natural flowers of *Asclepias syriaca*, and (ii) controlling yeast inoculation in artificial nectar. They consistently obtained that *Apis mellifera* did not discriminate, judged by number of visits or movements to each treatment, among nectar, or artificial syrup, with and without yeasts. Albeit they assessed microscopically presence of yeast cells in nectar of flowers exposed to natural insect visitation, we can not unerringly conclude that their experiment compared yeast-free against uniformly yeast-contaminated flowers. Variance among open flowers could increase measurement error because yeast abundance may be extremely variable within a single plant (Herrera *et al.*, 2008; Pozo *et al.*, 2009) and consequently reduce differences among the two analyzed categories. Apart from this, volatiles emissions by certain yeast species are attractant to beetles (Graham *et al.*, 2011), and consumption of fairly large amounts of nectar high in alcohol, as produced by a specialized fungal fermentation community, has been reported in

other floral visitors such as wild treeshrews visiting bertam palm plants (Wiens *et al.*, 2008), or wasps consuming nectar of *Epipactis* orchids (Ehlers and Olesen, 1997).

This study raises the question of why bumblebees preferred nectar supplemented with yeasts. It has been long known that the components that mediate in bees-flowers interaction are extremely complex, and that bee use combinations of visual, olfactory and sensorial cues to find appropriate flowers (reviewed in Dotterl and Vereecken, 2010). Yeast-contaminated nectar might be thus characterized by multicomponent floral signals potentially attractant to bees. In fact, we have detected differential bumblebee response to different yeast species. One possible explanation is that the bees directly detect the presence of the yeast cells themselves (Corbet, 1984; cited by Elhers and Olesen, 1997), since the three yeast species used in our assays differ in mean cell size and overall morphology of cell groups. In nectar-like sugar solutions, cells of *M. gruessii* occur as "airplane" tridimensional aggregates formed 3-4 cells around 25 μm long (see Fig. 1 in Herrera *et al.* 2011 Mol Ecol Clonality), which are about three- and two times larger, respectively, the size of *C. bombi* and *M. reukaufii* cell groups (MIP, *pers. obs.*). Another mutually nonexclusive explanation for bumblebee differential response to yeast species is that yeasts are detected and distinguished by the emission of ethanol or other volatiles arising from fermentation (e.g., Nout and Bartelt, 1998). We did observe bumblebees often becoming "sluggish" following consumption of large volumes of yeast-inoculated syrup, although we did not measure fermentation activity in our artificial nectars. Even so, it appears that *M. gruessii* (slowest fermentative capacity, largest size) and *C. bombi* (fastest fermentative capacity, smallest size) might be detected either by size or volatiles emission, and *M. reukaufii* by the combined effect of the former two, according to interspecific differences in glucose fermentation rate tests (MIP *et al.*, unpublished data). Consistent with this hypothesis is our finding that *M. reukaufii* supplementation resulted in the strongest preference response by bumblebees.

Yeast inhabiting floral nectar may also provide, or represent by themselves some sort of reward to pollinators. For example, nectar yeast metabolic action may

reward pollinators by warming the flowers of winter-flowering plants (Rands and Whitney, 2008; Herrera and Pozo, 2010), and yeast cells may provide a protein or amino acid source to bumblebees. In fact, protein supplementation is crucial for colony maintenance, especially during larval development, and supplementation of bee diet with yeast extract results in higher bee development in honeybees (Somerville, 2005) and *Scaptotrigona* stingless bees (Fernandes da Silva and Zucoloto, 1990).

***Potential consequences to plant-pollinator interactions***

Bumblebee preference for yeast-contaminated nectar, as we have found in this study, should be confirmed in field conditions. Bumblebees are facultative endothermic bees, able to fly at ambient temperatures as low as 5ºC (Heinrich, 2004) making them one of the most important pollinators in cool and early flowering species of temperate regions (see e.g. Mosquin, 1971). Increasing the number of visits in yeast-contaminated flowers may have important consequences for plants and yeasts because could result in rapid proliferation and spread of nectar yeasts in natural populations of flowers. In addition, preference for yeast-contaminated flowers may condition the evolution of possible defensive strategies of plants against nectarivorous yeasts. It has been hypothesized that plant would be interested in evolve substances deterring "nectar robbers", yeasts likewise, as they convert nectar in a less valuable reward for legitimate visitors as plant pollinators (Adler, 2000). However, we have obtained that yeast presence in nectar attracted pollinators as strongly as increasing two-fold the sugar concentration. Pollinator increased frequency of visits and time per flower may increase pollen removal, flow, and deposition (Harder and Thomson, 1989); thus potentially affecting plant fitness. The net fitness consequences of pollinator attraction and reward modification in those species or individuals harbouring nectar yeasts in their flowers are currently unexpected.

Nectar chemistry strongly contributes to increase plant attractiveness for flower-visiting insects (González-Teuber and Heil, 2009), and yeasts inhabiting floral nectar are best known so far for their demonstrated role as nectar sugar

consumers and the ensuing alterations they induce in nectar sugar composition and within-plant variance in nectar features (Herrera *et al.*, 2008, de Vega *et al.*, 2009). Several theories have been proposed to explain forager preference for certain nectar traits, as those models advocated by energy gain expectations (optimal foraging theory) or those describing a consistent preference for a given type of nectar reward, irrespective of its reward value (innate constancy behavior). The estimates for the metabolic costs of flight for bumblebees suggest that flying bumblebees have one of the higher metabolic rates recorded in any organism, being a 75% higher than in hummingbirds (Goulson, 2010). According to optimal foraging models, foraging bees are expected to maximize energy gain expectations in the minimum possible time (Heinrich, 2004), and our results concerning yeast-mimicking detailed effect on nectar concentration are consistent with this expectation. Consequently, we have obtained a significant preference for more concentrated sugar solutions, in agreement with previous investigations (Cnaani *et al.*, 2005; Whitney *et al.,* 2008). In the case of groups of artificial flowers with the same mean but different variances, we would expect bees to leave a group of flowers following frequent encounters with low-reward flowers. Our risk-perception tests are also in some agreement with the departure rules used by foragers, as we have obtained that bees tend to leave earlier the most "risky" flower patches (Herrera *et al.*, 2009). Plain sugars solutions of the three sugars, even at the same concentration, can be processed differentially by bumblebees, as well. Bumblebees need to cleave the large sucrose into glucose and fructose usable molecules, and fructose can be used to increase flight muscle temperature by a mechanism known as "substrate recycling" (Leite *et al.,* 1988). Sweetness also differs among the three sugars, being lower in glucose solutions. Nevertheless, we were unable to detect bumblebee clear discrimination for any of the three examined sugars, probably because we equalized energy gain among solutions by using the same weight of sugar, and forager preference rules seem to follow the energetic value of the three sugar molecules (Wells *et al.*, 1992).

In conclusion, albeit bumblebees seemed to maximize their energy gain and yeast-inoculated nectars constitute impoverished solutions from a sugar content viewpoint, we have obtained that bumblebees consistently preferred yeast-inoculated flowers. Nectar yeasts effect on pollinator behaviour, whereas it would be supported by field data, open novel avenues of research for nectar inhabiting yeasts and their role in the reproductive success of different plant species, growing in different ecological contexts. In particular, our results suggest that in the real world, where yeast presence and nectar sugar composition and concentration are correlated across plants and flowers (Herrera *et al.* 2008, de Vega *et al.* 2009), foraging bumblebees will probably face a trade-off whereby they should balance their innate preference for yeast-containing nectars against energetic optimality criteria leading them to prefer the energetically most rewarding flowers. Understanding how bumblebees and perhaps other pollinators manage this possible conflict may shed further light on the ecology and evolution of the tripartite interaction linking plants, pollinators and specialized nectar yeasts.

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## Discusión general

El objetivo principal de esta tesis doctoral ha sido el análisis del papel que ciertos elementos "invisibles", los microorganismos que habitan el néctar floral -más concretamente levaduras-, juegan en la dinámica del mutualismo planta-polinizador. En su línea más descriptiva, esta memoria reduce la carencia de datos disponibles sobre patrones de distribución y abundancia de levaduras en el néctar floral, y avanza en la identificación de los factores que determinan la composición y estructura de sus comunidades en condiciones naturales. Otros trabajos realizados en paralelo (Herrera *et al.* 2008; Herrera y Pozo, 2010), que demuestran cambios físicos y químicos inducidos en el néctar por el metabolismo de las levaduras, permitieron considerar a estos microbios como consumidores de este recurso (es decir, nectarívoros), ya que provocan cambios en la concentración y composición de azúcares y aminoácidos (Herrera *et al.* 2008; Peay *et al.*, 2011), así como en la temperatura del néctar (Herrera y Pozo, 2010). Por eso, en la parte final de esta tesis se valora experimentalmente el grado en el que los insectos polinizadores podrían advertir directa o indirectamente la presencia de levaduras nectarívoras en el néctar floral, modificar su elección floral, y con ello su función polinizadora.

***Patrones de distribución y abundancia de levaduras en el néctar floral***

A pesar de que la calidad final del néctar como recompensa floral se ha relacionado con la presencia de levaduras en algunas especies (Herrera *et al.*, 2008), la importancia de la interacción ternaria "planta-polinizador-levadura" depende necesariamente de la generalidad de la presencia de levaduras en el néctar floral de las angiospermas. Este hecho contrasta con la carencia de datos cuantitativos sobre la presencia de levaduras en distintos grupos de plantas superiores. Así, el primer reto abordado en esta tesis doctoral ha sido el estudio microscópico del néctar de múltiples especies de plantas de una misma región, con distintos grados de relación filogenética, objetivo abordable en una región con elevada diversidad vegetal como es el Parque Natural de Cazorla, Segura y las Villas (Capítulo 1). El estudio

detallado de la incidencia de levaduras en el néctar floral precisa indispensablemente de la caracterización de su frecuencia (porcentaje de muestras en la que se observan levaduras), así como de una valoración lo más precisa posible de su abundancia, por el efecto denso-dependiente que estas levaduras tiene sobre la calidad del néctar como recurso (Herrera *et al.*, 2008; Herrera y Pozo, 2010). Esta memoria incluye la lista más amplia de especies vegetales, hasta la fecha, para la detección y cuantificación de levaduras en el néctar floral en una misma región de estudio, con un total de 105 especies, pertenecientes a 25 familias y 15 órdenes.

Para el caso concreto de la frecuencia, se han realizado esporádicamente estimas sobre la prevalencia de muestras con levaduras en el néctar floral de algunas especies. Este estudio aporta una cifra de frecuencia del fenómeno contaminación del néctar con levaduras del 40% de las especies estudiadas, en concordancia con las estimas obtenidas en localidades de Asia, India, Sudáfrica y el suroeste de la Península Ibérica (Jimbo, 1926; Sandhu y Waraich, 1985; de Vega *et al.*, 2009; Herrera *et al.*, 2009). Nuestra cifra es también parecida, aunque ligeramente inferior, a la frecuencia de levaduras en el néctar de plantas de la Península de Yucatán (60% de las especies estudiadas; Herrera *et al.*, 2009), esta región mexicana supone el único ambiente subtropical de los estudiados hasta la fecha. Respecto a este valor máximo de presencia de levaduras, sería esperable encontrar valores más bajos en ambientes con marcada estacionalidad, ya que en éstos no se encuentran muchas flores disponibles para su colonización durante una importante parte del año, o bien en ambientes con condiciones climáticas más extremas durante todo el año, como zonas de latitudes muy bajas o bien ambientes desérticos.

Nuestro estudio ha permitido apreciar que la presencia de levaduras en los néctares florales es un fenómeno extendido en diversas familias de plantas, respondiendo afirmativamente a nuestro interrogante inicial acerca de la prevalencia/generalidad del fenómeno a nivel interespecífico, y demostrando también que las distintas especies muestran unos valores desiguales de frecuencia de levaduras en el néctar para una misma región. La búsqueda de patrones filogenéticos para la presencia de levaduras en el néctar floral ha sido realizada

previamente para un total de 27 familias de plantas en el centro de Alemania, obteniéndose en ese caso correlaciones significativas entre la presencia de levaduras y distintas propiedades del néctar asociadas a este nivel taxonómico, como pH, composición de azúcares (cociente glucosa + fructosa/sacarosa) y concentración de azúcares totales (Brysch-Herberg, 2004). Dado que las diferencias entre familias en relación a la frecuencia de levaduras en el néctar resultan ser poco informativas en nuestro estudio, y que la obtención de datos sobre las características físico-químicas del néctar para la totalidad de las especies incluidas requeriría un esfuerzo de muestreo y análisis que supera el ámbito de este estudio (Herrera et al., 2006), no se ha incluido aquí el análisis de las propiedades físico-químicas del néctar y su correlación con las diferencias mostradas entre especies para la abundancia de levaduras en el néctar floral.

En relación a la abundancia alcanzada por las levaduras en el néctar de distintas especies, en la región de Cazorla se ha obtenido un promedio por especie de planta de unas $10^3$ células por microlitro de néctar, valor que resulta algo inferior a los valores medios obtenidos para otras zonas geográficas, con distintas condiciones bioclimáticas, como Yucatán, Sudáfrica y el suroeste de la Península Ibérica (Herrera *et al.*, 2009; de Vega *et al.*, 2009). En este caso también se detecta que distintas especies de planta de una misma familia pueden presentar diferencias significativas en la abundancia de levaduras en su néctar, variación interespecífica que limita la importancia de patrones filogenéticos a escalas más generales para la determinación de la abundancia de levaduras en el néctar (Capítulo 1; Figura 3). Ante estos resultados, y ante la carencia de datos sobre patrones de distribución de las levaduras florales, cabe preguntarnos en este punto qué tipo de factores determinan la incidencia de levaduras en el néctar floral de las distintas especies en condiciones naturales.

A nivel intraespecífico, la incidencia de levaduras en el néctar (frecuencia y abundancia) podría relacionarse con gradientes ambientales como son la variación a lo largo de un gradiente altitudinal, o bien a aspectos temporales ligados en el caso de *H. foetidus* a una época de floración dilatada, al igual que sucede con los patrones

de distribución de numerosos macroorganismos (Gómez y Lunt, 2007), y que resultan bien definidos en la región de Cazorla. Trabajando sobre la hipótesis anterior, se detectaron diferencias extremas tanto en la abundancia como en la frecuencia de levaduras en el néctar de una misma especie de planta, en función de la fecha de muestreo y el lugar elegidos. Sin embargo, estas diferencias no han seguido patrones consistentes para los dos años de muestreo en función de los gradientes establecidos por las variables altitud y época de floración (Capítulo 2), lo que apunta a la existencia de variaciones supraanuales en cuanto a la distribución y/o abundancia de las levaduras del néctar en nuestra región de estudio.

En cuanto a la variación temporal de la presencia de levaduras en el néctar de *H. foetidus* dentro de una misma población, encontramos una frecuencia creciente desde el principio del periodo de floración hasta el momento de máxima floración de la especie en esa localidad. Al tratarse de una planta de floración invernal, este patrón se halla íntimamente ligado al principio del periodo de actividad de sus principales visitantes (abejorros). También se ha avanzado, en relación a la importancia relativa de las distintas escalas espaciales estudiadas (gradiente altitudinal, poblaciones, individuos, flores y nectarios), encontrando que las diferencias espaciales en presencia de levaduras en el néctar floral se producen, principalmente, dentro de la escala intra-floral. La escala a la que se produce esta variación apunta como mecanismos causantes a factores ecológicos que operen específicamente a este nivel. A pesar de que para establecer una relación de causalidad entre las variables ambientales exploradas en este trabajo y la presencia de levaduras en el néctar sería necesaria la realización de estudios experimentales, nuestros resultados permiten avanzar en la comprensión de los mecanismos subyacentes a los patrones de distribución de las levaduras del néctar. Nuestros resultados corroboran que la composición y actividad de los polinizadores, así como la temperatura ambiente media, son factores correlacionados con la frecuencia y abundancia de levaduras obtenidas en promedio para cada uno de los muestreos realizados. En la presente memoria se analiza la existencia de posibles correlaciones entre el tipo de polinizador dominante y la frecuencia y abundancia de

levaduras en el néctar para diversos muestreos de *H. foetidus* (Capítulo 2), así como para un 12% de las especies incluidas en el estudio interespecífico del Capítulo 1 (Capítulo 3). En ambos casos se observó que, las poblaciones y las especies con mayor carga de levaduras en su néctar son visitadas fundamentalmente por abejorros, al menos para una buena parte de especies de la región de Cazorla (ver Herrera *et al.*, 2009 para un análisis con otras regiones geográficas). Este resultado concuerda con las conclusiones de trabajos experimentales previos realizados en la misma zona de estudio (Canto *et al.*, 2008). Sin embargo, en otras regiones, como Sudáfrica o Norteamérica, las levaduras del néctar floral son transferidas preferentemente por aves (de Vega *et al.*, 2009; Belisle *et al.*, 2011). Por otro lado, los patrones de incidencia de levaduras en el néctar no han logrado relacionarse con otros factores bióticos, como la densidad floral en estas poblaciones, factor que sí se correlaciona con la distribución de levaduras en distintas plantas de *M. aurantiacus* en California (Belisle *et al.*, 2011).

Los resultados obtenidos para la presencia de levaduras en *H. foetidus* permiten, además, guiar el diseño de futuros estudios sobre la distribución de levaduras del néctar floral, así como advertir de su presencia a cualquier estudio cuyas conclusiones se hallen ligadas a las condiciones del néctar floral. En primer lugar, los datos ofrecidos conducen a la conclusión de que los patrones de incidencia de levaduras siguen patrones generados a escalas más pequeñas de las estudiadas habitualmente y que, al dirigirse por factores tan azarosos como la ruta de los polinizadores, deben ser escalados de manera acorde en su diseño. En segundo lugar, la variación existente en la escala intra-individual para la presencia de levaduras en el néctar provoca la existencia de una importante fuente de variación entre flores de una misma planta para las características del néctar, varianza que opera sobre la capacidad de plantas y polinizadores de ejercer selección sobre las características de este recurso, incluyendo la propia presencia de estos microorganismos (Herrera, 2009).

Una parte importante de los estudios disponibles sobre microbiología floral no distinguen los habitantes específicos del néctar de otros presentes en diferentes partes florales como la corola. Además, gran parte de estos trabajos son previos al uso de las técnicas moleculares de identificación de microorganismos (revisado en el Capítulo 3). Uno de los objetivos fundamentales de esta tesis doctoral ha sido, por lo tanto, lograr una identificación rigurosa de las especies de levaduras integrantes del néctar floral. La utilización de métodos de identificación independientes de cultivo, realizados sobre mezclas de ADN de diversos organismos, como RAPD-PCR (Randomly Amplified Polymorphic DNA PCR) y TGGE (Temperatura Gradient Gel Electrophoresis), han dado lugar a estimas de biodiversidad poco robustas frente a los resultados obtenidos por aislamiento y secuenciación (Herzberg *et al.*, 2002; Gadanho y Sampaio, 2004). La creciente competitividad de los costes de las técnicas de secuenciación masiva a partir de la subunidad pequeña del rARN (Tringe y Hugenholtz, 2008) convierten este método en una nueva y posible opción en estudios de biodiversidad de organismos, más aún en el caso de los organismos microscópicos, en los que su elevado número y pequeño tamaño suponen dificultades adicionales para su muestreo (Novais y Thorstenson, 2011). Sin embargo, aún resta por comprobar su robustez en la identificación de mezclas desiguales de organismos contenidos en pequeños volúmenes de muestra, como las que tienen lugar en el néctar floral.

Por ello, en esta tesis la identificación de las levaduras del néctar ha sido realizada por aislamiento en placa seguido de identificación por métodos moleculares (secuenciación unidad transcripcional ARN 26s). La gran cantidad de secuencias disponibles en GenBank (http://www.ncbi.nlm.nih.gov/genbank) para el dominio D1/D2 de la unidad transcripcional del ARN 26S ha permitido identificar con un alto porcentaje de verosimilitud las cepas aisladas en el néctar de 24 especies de plantas, detectándose una riqueza en especies de las comunidades de levaduras del néctar llamativamente baja, ya que el 85% de los aislamientos pertenecieron a un par de especies del género *Metschnikowia*, *M. reukaufii* y *M. gruessii*.

Sólo se ha aislado una cepa cuya secuencia no ha sido asimilable a ninguna especie conocida, si bien tanto las pruebas moleculares como las que atienden al perfil fisio-morfológico de las especies conducen a su inclusión dentro del género *Metschnikowia.*

A partir de los datos sobre las especies que finalmente componen las comunidades de levaduras del néctar se ha abordado la valoración de la riqueza específica de estas comunidades. Esta valoración se ha realizado, por primera vez, mediante un doble enfoque, que comprende a las especies de plantas (unión artificial de hábitats) así como del néctar de una flor individual como unidad de estudio (Capítulo 3). Para su comparación con otros estudios disponibles, frente a los cuales existirá un desigual esfuerzo de muestreo, se han empleado métodos de rarefacción (Hughes *et al.*, 2001). La curva de acumulación de especies de levaduras del néctar obtenida en relación al número de plantas estudiadas para Cazorla muestra una gran similitud con aquella obtenida a partir de las identificaciones de levaduras del néctar en 25 especies de plantas de Alemania (identificadas por secuenciación, datos extraídos de Herzberg *et al.*, 2002). En cuanto a la riqueza específica obtenida para una muestra individual de néctar (promedio de 1-2 especies de levadura por muestra), supone la única estima disponible de riqueza específica a esta escala para las comunidades florales. Sin embargo, sí existen datos comparables para las comunidades de levaduras asociadas a los tejidos necróticos de cactus del género *Stenocereus* en zonas desérticas (Starmer *et al.*, 2005), cuyo promedio por planta individual (unidad mínima de muestreo en este caso) es también inferior a 2 especies.

Para aquellas especies de levaduras cuyo hábitat prioritario es el néctar floral (como *Metschnikowia*, ver Introducción), su ambiente consiste en una serie de unidades discretas (flor individual, o nectario, en caso de haber varios y encontrarse éstos separados físicamente) potencialmente colonizables. Por otro lado, la naturaleza efímera de las flores, unida a la propia dinámica de producción y consumo del néctar por parte de organismos nectarívoros, hacen que la persistencia de las levaduras en este medio dependa fundamentalmente de la dinámica de

fenómenos de inmigración, crecimiento, dispersión y extinción (Kinkel, 1997; Herrera *et al*., 2012). Además, según la hipótesis del "filtrado de especies", la alta concentración de azúcares, y/o por la presencia en una amplia proporción de plantas de compuestos secundarios (Adler, 2000), podrían filtrar el elenco de potenciales colonizadores por procesos de resistencia diferencial (Herrera *et al*., 2010). La comentada pobreza en especies de las comunidades de levaduras del néctar, unida al hecho de que sólo unas pocas especies, del mismo género, presenten una gran ubicuidad en las numerosas muestras de néctar analizadas (a pesar de los presumibles cambios de condiciones bióticas y abióticas en las diversas localizaciones y plantas de recogida a lo largo de este estudio, ver también Capítulo 2), sugieren como fundamental el análisis de los procesos migratorios, así como de los condicionantes fisiológicos, como factores influyentes en la formación de las comunidades de levaduras del néctar floral en condiciones naturales. Es importante resaltar que el muestreo efectuado en esta tesis considera específicamente como potenciales fuentes externas de levaduras hacia el néctar al filoplano directamente en contacto con la muestra de néctar, el aire que rodea la planta, y los polinizadores que la visitan, lo cual supone un enfoque novedoso y más específico que permite detectar la importancia desde un punto de vista cuantitativo de los procesos de inmigración, así como el grado de intercambio de especies entre los distintos microambientes.

La frecuencia de levaduras es muy alta en las denominadas "fuentes externas", especialmente en las glosas de los insectos visitantes. Sin embargo, las dos especies de plantas estudiadas se diferencian ampliamente en la frecuencia de levaduras en el néctar, siendo ésta equiparable a la hallada en las glosas de los insectos en *D. obscura* y nula en el caso de *A. baetica*. Las diferencias entre especies de plantas se extienden también a la importancia de los insectos como vectores de levaduras al néctar. Así, mientras que las visitas de los insectos resultan ser claves para encontrar levaduras en el néctar de *D. obscura*, en el caso de *A. baetica* sólo se han hallado levaduras en el néctar de flores excluidas experimentalmente de las visitas de los insectos. La especie de *A. baetica* destaca por tanto como un caso

bastante particular, ya que se caracteriza por poseer levaduras en menos de la mitad de las muestras de néctar analizadas (20-45%, periodo 2008-2010), y presentar en cambio unas tasas muy altas de visitas en sus flores, hecho que podría limitar el rango temporal necesario para su proliferación.

En cuanto a la identidad de las especies de levadura, se ha corroborado que las levaduras de filoplano y aire conforman un conjunto de especies relativamente amplio, y de carácter más generalista, mientras que las piezas bucales de las abejas contienen un conjunto de especies más limitado, en el que se incluyen varias especies osmotolerantes (Lachance *et al.*, 2006; Herrera *et al.*, 2010). El néctar y el polen presentan, respecto a las mencionadas fuentes externas, una riqueza en especies llamativamente menor, dos especies cada uno. Estas dos especies suponen, en la población de *D. obscura*, un subconjunto empobrecido respecto a las potenciales especies colonizadoras presentes en las glosas de las abejas. En el caso de *A. baetica*, las levaduras aisladas del néctar provienen de flores no visitadas por abejas, y pertenecen a especies de índole más generalista, pero sobre todo adaptadas a altas temperaturas, lo cual podría ser un factor decisivo en la conformación de comunidades de levaduras florales en una planta de floración estival.

En relación a los procesos fisiológicos que podrían determinar las especies de levaduras finalmente halladas en las comunidades del néctar, se ha detectado que las levaduras del néctar difieren de las especies de levadura asociadas a aire y filoplano en sus patrones de utilización de fuentes de carbono y nitrógeno. Sin embargo, y contrariamente a nuestra hipótesis inicial del "filtrado de especies", el género *Metschnikowia* no ha mostrado una resistencia netamente superior respecto al resto de especies ante altas concentraciones de azúcar (ver Herrera *et al.*, 2012 para datos de concentración de azúcares de *D. obscura* y *A.baetica*), ni ante los compuestos secundarios presentes en estas especies (ver caracterización de las especies de estudio en la Introducción). Aun así, sí se ha detectado para las cepas del género *Metschnikowia* una tasa de crecimiento muy superior en un amplio espectro de temperaturas, factor que se postula como un factor decisivo en la constitución de las comunidades de levaduras asociadas al néctar floral, un medio

caracterizado por su marcado carácter efímero. Otros estudios ya habían citado el papel del rango de tolerancia de las distintas especies de levadura a la temperatura sobre la conformación de comunidades de levaduras asociadas a plantas (Lachance, 2006; Sampaio y Gonçalves, 2008), si bien estos trabajos valoran principalmente las resistencias a valores extremos.

La importancia otorgada en esta tesis a la osmotolerancia y resistencia a compuestos defensivos como procesos para la conformación de comunidades de levaduras en el néctar podría verse ampliada al incluir experimentalmente la interacción entre la composición y concentración de azúcares y la temperatura de crecimiento (Chin *et al*., 2010). La varianza en la composición y concentración de azúcares dentro de las especies de plantas estudiadas (Herrera *et al*., 2011), así como las dificultades técnicas asociadas a la cuantificación de los compuestos secundarios, no han permitido recrear de un modo más complejo las características del néctar en nuestras plantas de estudio. No obstante, la escasa magnitud alcanzada por los citados efectos por separado permite concluir que, en caso de existir, los efectos de las interacciones tendrían una relevancia moderada.

A pesar de la comentada tendencia general para las cepas de las principales levaduras del néctar, *M. reukaufii* y *M. gruessii*, para las que se cuenta con numerosos aislamientos, se observa la existencia de una gran variabilidad fenotípica entre cepas de la misma especie. Este es un resultado muy interesante para estudiar la base genética de la diversidad fenotípica observada, teniendo en cuenta que estas especies carecen de reproducción sexual -uno de los motores propulsores de variabilidad genética entre individuos de la misma especie- en condiciones naturales. Inesperadamente, los niveles de diversidad genética observados en las poblaciones clonales de *M. gruessii* no resultan inferiores a los niveles exhibidos por organismos con reproducción sexual (Herrera *et al*., 2011). Concretamente, los distintos sustratos de procedencia (filoplano, glosas, néctar y polen, caracterizados por contrastadas presiones ambientales, Ngugi y Scherm, 2006) muestran distintas estimas de diversidad genotípica, por lo que la selección divergente podría postularse como el principal mecanismo propulsor de diversidad genética en este

tipo de organismos. A las ya nombradas diferencias entre genotipos de la misma especie de levadura en base a la variación en su secuencia nucleotídica, pueden superponerse cambios epigenéticos, en base a procesos de metilación del ADN. Estos procesos de metilación, en el caso de *M. reukaufii*, parecen contribuir de manera efectiva a ampliar la plasticidad fenotípica de esta especie, ampliando sus límites de tolerancia en concentraciones de azúcar superiores al 40% y en medios compuestos por fructosa, que les suponen un mayor estrés (Herrera *et al*., 2012). Curiosamente, ambos extremos pueden ser encontrados con frecuencia en el néctar floral en condiciones naturales (Stiles y Freeman, 1985; Herrera *et al*., 2006; 2008; Canto *et al*., 2007; 2008).

***Las levaduras del néctar floral y su efecto sobre el forrajeo de los abejorros***

El estudio de las levaduras del néctar, y sus efectos sobre las características del mismo, añade una nueva e inexplorada faceta al estudio de la relación planta-polinizador, la importancia de las levaduras nectarívoras y su efecto sobre los consumidores finales del néctar contaminado, los polinizadores. Ya que esta consecuencia, y los mecanismos por los que podría producirse, no han sido estudiados hasta la fecha (a excepción del trabajo puntual de Kevan *et al*., 1988, con *Apis mellifera*), el último aspecto explorado en esta tesis es la respuesta de *Bombus terrestris* ante la presencia de levaduras en medios que simulan las características del néctar contaminado. Los ensayos se han realizado en condiciones experimentales, con colmenas cautivas, para reducir la multiplicidad de factores experimentales que no hubiera sido posible controlar en condiciones de campo. Se ha encontrado que individuos de *B. terrestris* muestran preferencia por los medios suplementados con levaduras, ya que visitan las flores artificiales con levaduras con una mayor frecuencia, y durante un periodo más largo, que las flores sin levaduras (Capítulo 5). Es importante señalar que, al realizar los ensayos con tres especies de levadura provenientes del néctar natural, ha podido detectarse que el efecto atractivo de las levaduras del néctar varía en función de la especie. Este resultado inesperado es particularmente interesante ya que la especie que provoca una mayor respuesta en

los insectos provenientes de colmenas cautivas es precisamente *M. reukaufii*, la principal levadura del néctar en condiciones naturales. Sin embargo, al recrear separadamente una serie de modificaciones químicas que las levaduras inducen en el néctar, tales como disminución de los azúcares totales, cambio en el tipo de azúcar predominante e incremento en la varianza entre muestras contiguas, únicamente se ha obtenido una marcada respuesta negativa a la disminución de la concentración total de azúcares, en la línea de estudios clásicos sobre la incidencia de patrones de eficiencia energética sobre el forrajeo de abejorros, insectos caracterizados por una alta tasa metabólica (Heinrich, 2004; Goulson, 2010).

Los datos ofrecidos en esta memoria en relación a la respuesta positiva de nuestros insectos modelo ante la presencia de las levaduras principales del néctar en la región, *M. reukaufii* y *M. gruessii*, permiten finalmente contextualizar la importancia ecológica de los resultados anteriores, comprobando así de manera empírica la afirmación inicial de que las levaduras del néctar constituyen una parte activa en la interacción planta-polinizador. Nuestros resultados arrojan luz sobre diversos condicionantes del comportamiento de *B. terrestris* en ambientes que, aunque artificiales, han sido diseñados para recrear datos "reales" en relación a densidad de levaduras del néctar (ver Capítulos 1 y 2), e identidad de las especies presentes (Capítulo 3, Herrera *et al*., 2010). Asimismo, los experimentos de disminución de azúcares totales y conversión del tipo de azúcar predominante responden a la variación natural inducida por levaduras nectarívoras (Herrera *et al*., 2008). Por ello, es posible afirmar la relevancia de los resultados obtenidos experimentalmente, y concluir que los datos ofrecidos suponen un paso inicial indispensable de cara a realizar ensayos en el campo sobre el efecto de las levaduras del néctar en el comportamiento de los polinizadores. Si los resultados obtenidos en el Capítulo 5 de esta memoria fueran corroborados por estudios en condiciones naturales, la presencia de levaduras, concretamente de aquellas habitantes más especializadas del néctar floral, como *M. reukaufii*, tendrían un efecto favorable sobre la atracción de insectos polinizadores, en un mecanismo complementario a la secreción del néctar por la planta. Resultaría indispensable, en un paso siguiente, valorar el efecto que el

incremento en el número de visitas a sus flores, así como en la longitud de las mismas, podría llegar a suponer para el éxito reproductivo de la planta.

### Nuevos interrogantes

La presente tesis avanza en el conocimiento de las levaduras nectarívoras en relación a diversos aspectos cuantitativos y cualitativos relacionados con la ecología de sus comunidades, y proporciona las primeras evidencias de capacidad de detección de estas levaduras por parte de los polinizadores. Ambos aspectos apoyan la necesidad de incluir estos microbios como tercer agente en el mutualismo planta-polinizador. Sin embargo, su elaboración también ha permitido el planteamiento de nuevos interrogantes, los cuales conforman líneas de investigación que serían de gran interés para el futuro.

Los sugerentes resultados del comportamiento diferencial de los abejorros cautivos en respuesta a la presencia de levaduras en el néctar confirman la utilidad del diseño de experimentos más complejos, en condiciones de campo, sobre el efecto de las distintas especies de levadura, mezclas de las mismas, y sus diversas abundancias, en el néctar y la elección floral de los polinizadores.

El análisis de las comunidades de levaduras del néctar floral también sugiere nuevas oportunidades de investigación. Sería de gran interés el estudio de los procesos por los que se conforman estas comunidades en otras especies de plantas con distintos visitantes florales y época de floración; dada la importancia descrita para los procesos de inmigración y para la temperatura de crecimiento en la fase de establecimiento. Además, resultaría de particular interés el estudio de los mecanismos asociados al desarrollo de comunidades de levaduras nectarívoras en los casos extremos que identifica este estudio, ya sea en especies de plantas en las que nunca se observan levaduras, como en aquéllas en las que son particularmente abundantes.

Por otro lado, la detección de compuestos tóxicos en el néctar y la determinación de su concentración, supone todavía un reto técnico para el estudio de los procesos de inhibición del crecimiento microbiano que es necesario superar

para poder experimentar de un modo más realista sus efectos junto con otras características del néctar como pobreza en nitrógeno y alta concentración de azúcar.

El estudio de los patrones interespecíficos sobre la presencia de levaduras demuestra la existencia de una gran variabilidad entre especies de planta, por lo que la inclusión de un número creciente de especies posibilitaría el estudio de la consistencia del patrón descrito. Por otro lado, esta tesis planteaba como hipótesis inicial que la filogenia podría influir sobre la presencia de levaduras a través de su efecto en las características del néctar, pero este componente no pudo ser analizado al no contarse con esta información para todas las especies de plantas cuyo néctar fue analizado microscópicamente. Por lo tanto, se plantea que el estudio de patrones de secreción del néctar, polinizadores y composición química de un número mayor de especies vegetales sería de gran interés para entender las diferencias en cuanto a la incidencia de levaduras en el néctar que tienen lugar entre especies de plantas.

Asimismo, en esta tesis se describe una gran variabilidad espacial y temporal en los patrones intraespecíficos para la presencia de levaduras. Los patrones descritos no fueron consistentes entre años, por lo que sería necesario ampliar el periodo de muestreo, así como dirigir el estudio hacia las principales escalas de variación, es decir, la escala intra-individual, para avanzar en el conocimiento de los mecanismos implicados en los patrones de distribución de las levaduras del néctar. Así, los datos sobre variables abióticas y bióticas que podrían afectar los patrones de incidencia de levaduras en el néctar, deberían ser analizados a escala de flor individual o nectario, coincidente con el patrón de variación descrito para las levaduras del néctar en esta tesis, y deberían ser tomados durante un periodo temporal más amplio.

Además, sería de interés la realización de estudios sobre patrones de distribución similares a los realizados en esta tesis para *H. foetidus*, planta de floración invernal, para otras especies de planta cuyo periodo de floración corresponda a diferentes épocas del año, y con ello, respondan a diferentes condiciones climáticas y frecuencia y abundancia de visitantes florales.

## Conclusiones

1. La presencia de levaduras en los néctares florales es un fenómeno ampliamente extendido en múltiples familias y numerosas especies de plantas.
2. En una misma región, distintas especies muestran valores muy desiguales de incidencia de levaduras en el néctar, aunque no se aprecian patrones filogenéticos claros en el conjunto de plantas estudiadas.
3. Para una misma especie de planta existen diferencias extremas tanto en la abundancia como en la frecuencia de levaduras dependiendo de la fecha y la localidad de muestreo. Estos patrones se relacionan con la composición y actividad de los polinizadores, y con la temperatura media del periodo estudiado.
4. Este estudio destaca que las mayores diferencias en presencia y abundancia de levaduras tienen lugar, principalmente, en la escala intra-individual, entre flores de una planta, y nectarios de una flor.
5. Las muestras de néctar alcanzaron el valor más bajo de frecuencia y riqueza en especies de levaduras, en comparación con las comunidades asociadas al aire, piezas bucales de los insectos y filoplano de la misma planta.
6. La mayor parte de los aislamientos de levaduras del néctar corresponden a dos especies del género *Metschnikowia*, *M. reukaufii* y *M. gruessii*. En condiciones naturales, la mayor parte de las comunidades del néctar son monoespecíficas.
7. Estas dos especies del género *Metschnikowia* se diferencian de las halladas en aire y filoplano en sus patrones de utilización de fuentes de carbono y nitrógeno.
8. Las dos especies del género *Metschnikowia* muestran una tasa de crecimiento muy superior respecto al resto de potenciales colonizadoras en un amplio espectro de temperaturas, característica fundamental en la constitución de comunidades de levaduras en un medio efímero como el néctar floral. Sin embargo, estas dos especies no presentaron una resistencia superior ante

los compuestos secundarios presentes en las dos especies de estudio, ni ante altas concentraciones de azúcar.

9. La presencia de levaduras modifica la conducta de *Bombus terrestris*, que muestra preferencia por el tratamiento con levaduras, ya sea en su número de visitas como en la longitud de las mismas. Este efecto es variable en su magnitud entre distintas especies de levadura, siendo precisamente *M. reukaufii* la especie que provoca una mayor respuesta en los insectos.
10. Y por último, la conclusión más amplia y de interés general que se deduce de todas las anteriores es la siguiente: por su frecuencia, abundancia y posible importancia de sus consecuencias ecológicas y evolutivas, las levaduras del néctar son un factor a tener en cuenta en lo sucesivo al estudiar las interacciones entre las plantas y sus insectos polinizadores.

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